BIOLOGICAL CONTROL OF *Pseudomonas avenae* WITH EPIPHYTIC BACTERIA ISOLATED FROM CORN PLANTS

BY

CARLOS ALBERTO LOPES

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Abstract of Dissertation Presented to the Graduate School
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CARLOS ALBERTO LOPES

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The interactions among bacterial epiphytes and pathogenic species in the soil or on the plant surface often result in suppression of plant disease. Epiphytic bacterial populations in the fluid collected from the whorl of corn (*Zea mays* L.) plants in the field were found to correspond to a lognormal distribution. Therefore, samples for qualitative and quantitative studies of populations in this ecological niche should be analyzed separately for each plant. From 179 bacterial cultures collected from corn plants in three locations in Florida, 34 were antagonistic in vitro to *Pseudomonas avenae*, the causal agent of bacterial leaf blight and stalk rot (BLBSR) of corn. Among the antagonistic cultures, 29 were fluorescent pseudomonads. The number of antagonists to *P. avenae* that were collected from sweet corn cv. Silver Queen, which is resistant to BLBSR, did not differ from that collected from the susceptible cultivar, Merit. There was no correlation between antagonism in vitro and ability to control the disease in plants in the greenhouse. Among all the bacteria, only cultures F-11 and F-24 of *Pseudomonas fluorescens* and culture U-46 of *Bacillus* sp. consistently controlled the disease on plants in the greenhouse. Infectivity titrations with *P. avenae* on corn plants that were protected and not protected with antagonist F-11 indicated that the control achieved with the application of the antagonist was equivalent to reducing the inoculum dose at least 1000 times at the 50% level of disease intensity (ED_{50}). In the field, F-11 controlled BLBSR as effectively as 100 ppm streptomycin when the treatments were applied immediately before inoculation with *P. avenae*. When treatments were applied 12 or 24 hours prior to inoculation, however, streptomycin did not control BLBSR, whereas F-11 reduced disease intensity more than 50%. Antagonist U-46 did not affect disease development in the field at any time of application. Disease control by F-11 was apparently related to its ability to colonize the whorl and be redistributed to new tissues of the plant. *Pseudomonas fluorescens* F-11 was recovered in large numbers from corn-plant whorls as long as eight days after it had been applied to plants in the field. Conversely, antagonist U-46 could not be recovered from the whorls at 84 hours after its application. The mechanism by which

F-11 antagonizes *P. avenae* has not been fully determined, but antagonism is presumed to act mainly through stimulation of host resistance or through the production of unstable or volatile antibiotic substance(s).

CHAPTER ONE
INTRODUCTION

Bacterial leaf blight and stalk rot (BLBSR), incited by *Pseudomonas avenae* Manns, was first reported as a corn (*Zea mays* L.) disease in the United States in 1945 by Johnson et al. (48). The disease has since been reported in Brazil, Japan, and the Philippines (14). Bacterial leaf blight and stalk rot can cause significant economic losses if susceptible corn varieties are grown under tropical weather conditions (37,83). Although resistance to BLBSR has been reported for some sweet corn cultivars (83), the level of resistance is not high enough to be considered as a sole method of control. The most common symptoms of the disease are long, narrow lesions on the leaves and a stalk rot at or just above where the ears are produced. The stalk rot consists of a dark-brown to black rot of the internodes, nodes, and base of the ear. The latter symptom is also known as shank rot (14,35,48).

Pseudomonas avenae has a wide host range within the family Poaceae (14). Long-term survival of the bacterium in Florida was found to occur in association with seeds and leaf tissues of vaseygrass (*Paspalum urvillei* Steud.) (37).

Gitaitis (36) observed that areas of water collection in sweet corn plants corresponded to areas with symptoms of BLBSR and that lesions developed mostly on leaves emerging from or in the whorl at the time of inoculation. Data from infectivity titration experiments, electron microscope observations, and autoradiography indicated that the whorl is the most important court for infection by *P. avenae* (36). The rapid growth of a corn plant constantly exposes the expanding lower part of the leaf to the pathogen. The tissue is vulnerable, unless an applied bactericide has the property of redistribution (36). Local application of a bactericide to the whorl temporarily protected plants in the field, thus corroborating the fact that the whorl is an important infection court (36).

Corn and other upright grasses possess anatomical characteristics that make them a very efficient system for catching and retaining water. Water from dew, guttation, mist, rain, and irrigation runs down the lamina carrying leaf leachates, microorganisms, and particles from the atmosphere (75). The fluid collects temporarily in the top funnel of furled leaves and in the cisterns formed by the collar and sheath of older leaves, providing a habitat for specific microbial populations.

The influence of leachates on plant-surface microorganisms is very complex. It is known, however, that they affect microbial populations either by stimulating or inhibiting their growth (38,87). Stimulation of fungal spore germination and growth has been usually regarded as nutritional and attributed to carbohydrates and amino acids present (18,38). Inhibition of microbial growth has been related to phenolic or terpenoid substances secreted by leaves (13). Therefore, it is conceivable that temporal changes of these inhibitory substances, as well as the concentration of nutrient exudates, may play an important role in the succession of microorganisms on the plant surface.

Frossard (31) reported that guttation fluids probably never represent chemically pure water. Organic (carbohydrates and amino acids) and inorganic substances in the guttation fluids are believed to be responsible for the sustenance of pathogenic and epiphytic bacteria on leaf surfaces.

Gitaitis (36) analyzed the fluid collected from the whorl of sweet-corn plants and found that it contained essential compounds for microbial growth, such as sugars, organic acids, and amino acids. The filtrate of this fluid was not inhibitory to one culture of P. avenae.

The terms epiphytic, phylloplane, resident, and leaf-surface have generally been used in the literature to refer to bacteria that are capable of living (i.e., multiplying) on plant surfaces. Those which may land on the leaf by chance and cannot grow there were called "casuals" by Leben

(55). For practical purposes, the definition of epiphytic bacteria presented by Hirano and Upper (43) will be adopted: ". . . those bacteria that can be removed from aboveground plant parts by washing" (p. 246). The other terms might also be used eventually with the same meaning.

The sweet corn-*P. avenae* pathosystem presents characteristics that make it an attractive model for biological control studies. First, there is a close contact between the putative antagonist(s) and the pathogen's propagules in the whorl before infection takes place. Second, since young plants are more susceptible to BLBSR than older ones and the latent period of the pathogen is short (3-4 days), screening for antagonistic bacteria can be done at the seedling stage. Therefore, one can work with a large number of plants in a short period of time. Third, sampling is not destructive, allowing quantitative and qualitative studies on microbial succession within the same plant.

A potential biological control agent for BLBSR of corn would be a microorganism that colonizes the whorl and creates an environment unfavorable to *P. avenae*. Some of the ways microorganisms could modify the environment, thus affecting disease development, are by producing metabolites which inhibit or affect the virulence of the pathogen or induce resistance of the host, or by competing for nutrients and/or infection sites with the pathogen. For biological control, naturally occurring resident microorganisms which have become adapted for survival and growth on the phylloplane of corn plants would be preferable, as compared to equally effective antagonists from other habitats. Introduced organisms are less likely to survive as long and, therefore, would need to be reapplied more frequently to the plants (13).

Because bacteria are the first colonizers of the leaf surface of many plant species (11), it is important to determine how epiphytic bacterial populations affect BLBSR development.

The main purposes of this study were to screen epiphytic bacteria isolated from corn leaves and whorls for their ability to antagonize *P. avenae* in vitro and in planta and to assess the distribution and survival of some of the components of the bacterial populations on corn plants as related to the biological control of BLBSR of corn.

CHAPTER TWO
LITERATURE REVIEW

Increasing interest in research with biological control of plant diseases is evident world wide, especially in these days when deterioration of the environment by agricultural chemicals has been a constant subject of concern. Cook and Baker (20) have defined biological control as ". . . the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (p. 60). Although this broad definition seems to be generally accepted by plant pathologists today (19), biological control will be used in this dissertation to mean microbial antagonism, i.e., control of one microorganism through another microorganism (24).

The utilization of bacteria to control plant diseases has been extensively reviewed (12,79,82,89). Most reports deal with the control of fungal organisms, although a few examples occur for control of bacterial diseases with bacteria. Little is known about the competitive relationships among saprophytic and pathogenic bacteria on plant surfaces, but there is good evidence that these interactions affect disease development or frost injury (11,22,23,32,40,45,50,55,60,63,77-79,85,86,88,89,91).

The mechanisms of antagonistic interactions of epiphytic bacteria with microorganisms and with the plant may be divided into four categories: 1, antibiosis; 2, competition for nutrients and for physical or biological sites; 3, predation or parasitism by other microorganisms; and 4, stimulation of host defense (10,11,13,15,89).

The most dramatic and economically successful example of biocontrol of a bacterial disease with a bacterium is the control of crown gall incited by *Agrobacterium tumefaciens* with the strain 84 of *A. radiobacter* (50,69,70). For treatment, plant parts are dipped or sprayed with high populations (10^8-10^9 cfu/ml) of the antagonist before planting. The mechanism by which *A. radiobacter* strain 84 controls the crown gall disease is believed to be mainly through production of agrocin 84, a plasmid-coded bacteriocin which either kills the bacterium or prevents the attachment of *A. tumefaciens* cells to the host receptor sites (70). An unknown additional factor was also suggested as being necessary for disease control, since the transfer of the plasmid coding for bacteriocin from strain 84 to another strain gave less satisfactory control by the transconjugants when compared to the parent strain (27). This additional factor may explain why bacteriocin-resistant strains of *A. tumefaciens* can still be controlled with A. radiobacter strain 84 (70). The host plant may also play a role in determining whether or not disease will develop (70).

One of the first reports of biological control of a plant disease with bacteria was in 1958 by Farabee and Lockwood (30). They found that 35 out of 40 yellow bacteria isolated along with Erwinia amylovora from cankers on apple and pear trees were antagonistic to E. amylovora in vitro. One of the antagonists appeared to produce an antibiotic that was inhibitory to the pathogen, whereas another antagonist apparently inhibited E. amylovora by increasing the acidity of the culture medium.

Goodman (39) isolated bacteria from apparently healthy apple buds which suppressed fire blight symptoms when they were mixed with a suspension of E. amylovora prior to inoculation of apple shoots. Acid formation within the host tissue was believed to be the mechanism involved in disease control. A later report by Goodman (40) indicated that a fire-blight protective effect, created by infiltration of apple shoots with avirulent strains of E. amylovora, unknown Erwinia-like bacterium, or Pseudomonas syringae pv. tabaci, was due to a host-response mechanism triggered by the antagonistic bacteria. Partial control of fire blight was also obtained in the greenhouse and in the field when a suspension of E. herbicola was applied to pear blossoms 24 hours prior to inoculation with E. amylovora

(74). The *E. herbicola* strain consumed all organic nitrogen in a culture medium and lowered its pH to a level inhibitory to the pathogen. An identical phenomenon was suggested to occur in the nectar in vivo; biological control was achieved by making the nectary glands an unfavorable environment for infection and colonization of *E. amylovora* (74).

A delay in the expression of fire-blight symptoms was obtained by McIntyre et al. (66) when they applied avirulent strains of *E. amylovora*, *E. herbicola*, and *P. syringae* pv. *tabaci* to pear shoots 24 hours before inoculation with a virulent strain of the pathogen. Delay of symptom expression also occurred when cell-free sonicates of both avirulent and virulent strains of *E. amylovora* were injected into pear shoots. The sonicates did not inhibit multiplication of the bacterium or affect virulence of *E. amylovora* in vitro, therefore suggesting that the mechanism involved in disease suppression is host dependent, probably by production of phytoalexin-like substances.

Thomson et al. (86) selected saprophytic bacteria capable of multiplying in pear flowers and tested them for their antagonistic effects against *E. amylovora*. Fire-blight incidence was significantly reduced when cultures of three fluorescent *Pseudomonas* and one *Erwinia* sp. were sprayed on plants in the field during bloom. None of the pseudomonas produced antibiotic compounds in vitro, whereas the saprophytic *Erwinia* produced bacteriocins, as detected by formation of zones of inhibition to 60 of 62 cultures of *E. amylovora*.

Control of fire blight comparable to treatment with 100 ppm streptomycin was achieved when cell suspensions of two strains of *E. herbicola* were applied to apple trees (9). Since only one of the two strains of the antagonist produced bacteriocin in vitro, the mechanism of control could not be attributed to this compound alone. Beer et al. (8) have found that bacteriocin-like substances produced by different strains of *E. herbicola* inhibited differently strains of *E. amylovora*.

Isenbeck and Schulz (47) tested 29 yellow bacteria isolated from blighted parts of rosaceous plants for antagonism to *E. amylovora*. Seventeen of these inhibited the growth of the pathogen in vitro. Three selected antagonists significantly controlled fire blight in *Cotoneaster bullatus* plants under controlled conditions. In some cases, disease control was better with the biocontrol agent (applied 24 hours before inoculation) than with streptomycin at 200 ppm or the synthetic bactericide trimethoprim at 200 ppm. In field trials, disease incidence was reduced about 20% following the application of the best antagonist. The mechanisms involved in disease control were not determined.

Reduction or prevention of bacterial-blight symptoms on rice plants in the greenhouse was achieved when *E. herbicola* was applied to leaves in mixture with the pathogen, *Xanthomonas campestris* pv. *oryzae* (45). The magnitude of the control was dependent on the antagonist/pathogen ratio. Application of heat-killed and streptomycin-treated cells of *E. herbicola* did not reduce bacterial blight, thus indicating that disease control was not due to spatial interference. The mechanism of antagonism was probably related to acid production, since *E. herbicola* lowered the pH of a liquid medium to levels inhibitory to the pathogen. No other substances toxic to *X. campestris* pv. *oryzae* were produced in solid or liquid media.

Foliar symptoms of bacterial canker of tomato could be significantly reduced, as reported by Echandi (26), if tomato seedlings were sprayed with an avirulent mutant, bacteriocin-producing strain of *Corynebacterium michiganense* pv. *michiganense* or with cell-free bacteriocin preparations before spraying with the virulent strain. Disease control was also achieved when the seedlings were sprayed with a mixture of virulent and avirulent, bacteriocin-producing mutant of the pathogen.

Averre and Kelman (4) suggested that a host-response mechanism explained the protection of solanaceous plants challenged with a mixture of different proportions of virulent and avirulent strains of *Pseudomonas solanacearum*. Kempe and Sequeira (49) also found that brown rot severity was reduced when budded potato tubers were treated with avirulent strains of *P. solanacearum* and with strains of *P. fluorescens* before challenging them with a virulent strain of the pathogen. They stated that the suppression of disease might be due to a systemic resistance induced in the host by the antagonists.

Several cotton phylloplane bacteria of the genera *Flavobacterium*, *Aeromonas*, and *Pseudomonas* protected cotton leaves from infection when applied prior to inoculation with *Xanthomonas campestris* pv. *malvacearum* (88). None of these epiphytes were antagonistic to the pathogen in vitro and did not prevent infection when post or coinoculated. It was concluded that the inhibitory factor is produced prior to the establishment of the pathogen and is, therefore, predominantly host dependent.

Erwinia carotovora subsp. *betavasculorum* was found to be antagonistic to *E. carotovora* subsp. *carotovora* during potato tuber soft rot development. Production of an antibiotic by the former was reported as being the mechanism of antagonism between the two pathogens (5).

Teliz-Ortiz and Burkholder (85) isolated two strains of *P. fluorescens* from bean plants, grown in the greenhouse, which were antagonistic in vitro to 17 of 19 bacterial plant pathogens of four genera. This bacterium protected bean plants in the greenhouse against infection by P. syringae pv. phaseolicola. Control was suggested to be due to the production of an antibiotic which was absorbed and transported upward in the plant.

Fewer and smaller lesions of bacterial blight of rice developed when epiphytic Pseudomonas or Erwinia were applied 24 hours after inoculation with X. campestris pv. translucens (73). No lesions developed when the antagonists were applied 24 hours before inoculation. Although the mechanism involved in blight control was not determined, it was believed to be related to abundant acid production or excretion of toxic metabolites that interfered with disease development.

A saprophytic bacterium from cherry leaf surfaces and having affinity to Erwinia spp. was found by Crosse (22) to reduce intensity of cherry leaf scar incited by P. syringae pv. morsprunorum. The effect of this antagonist was comparable to reducing the inoculum concentration on artificial inoculations. Since no antibiotic production could be demonstrated, it was suggested that antagonism was due to nutrient competition during intravascular growth phases or due to stimulation of host response through metabolites secreted by the epiphyte.

Kloepper and Schroth (53) reported that the ability of plant growth-promoting rhizobacteria (PGPR) to induce increased potato plant growth was due to displacement of certain root-colonizing microorganisms, related in part to antibiosis. Mutants that had lost the ability to produce the antibiotic did not induce plant growth increase. All 16 PGPR tested had antibiotic activity in vitro against *E. carotovora* subsp. *carotovora* and other rhizosphere bacteria. Kloepper (51) confirmed later the antagonistic effect of PGPR to *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atrospetica* in potato seed pieces. He stated that antagonism could also be due to siderophore production or other bacterial metabolites.

One of three strains of *Bdellovibrio bacteriovorus* isolated from soybean rhizosphere was particularly effective in reducing localized and systemic symptoms of bacterial blight when it was mixed with *P. syringae* pv. *glycinea* prior to inoculation of soybean leaves (78). *Bdellovibrio bacteriovorus* is known to penetrate the cell wall of the host bacterium and multiply within it to a point that results in the release of new cells upon collapse of the host membranes.

A yellow bacterium, isolated from soybean leaves infected with *P. syringae* pv. *glycinea*, was reported to inhibit lesion development of bacterial blight of soybeans in the greenhouse when applied to leaves and buds in mixture with the pathogen (77). Antibiosis was suggested to explain the phenomenon, since the number of viable cells of the pathogen decreased on the plant with time, as compared to the control, when the ratio of antagonist/pathogen increased. The presence of this antagonist was believed to affect the population of the resident phase of *P. syringae* pv. *glycinea* on soybean buds and leaves.

Chakravarti et al. (16) found that 25 of 358 bacterial cultures isolated from terminal buds of field-grown soybean plants inhibited the growth of *Pseudomonas syringae* pv. *glycinea* in vitro. It was suggested that the presence of the antagonistic bacteria could affect the detection of populations of the pathogen in plant buds in the field.

Frost damage incited by ice nucleation-active (INA) strains of *P. syringae* and *E. herbicola* could be reduced in a growth chamber by spraying corn seedlings with a culture of non-INA *E. herbicola*, isolated from corn leaves, 24 hours prior to application of the INA bacteria (62). No evidence of antibiotic or bacteriocin production by the antagonist could be detected in vitro against the two INA bacteria. Therefore, other mechanism(s) might be involved in this specific interaction. Lindow (59) had demonstrated earlier that only 35 out of 60 bacterial cultures that reduced colonization of ice nucleation-active *P. syringae* and *E. herbicola* on leaf surfaces actually produced antibiotics to these two species in vitro. Furthermore, it was found that mutants of the strains that no longer produced either fluorescent or nonfluorescent inhibitory compounds in vitro equally prevented growth of *P. syringae* on corn leaves and reduced frost injury to seedlings (59). Field experiments carried out by Lindow et al. (63) confirmed that frost damage incited by INA bacteria can be reduced by spraying plants with antagonistic bacteria.

Twenty-five cultures of fluorescent pseudomonads were tested by Colin et al. (17) for antagonism in vitro against *P. syringae* pv. *tomato*, *E. carotovora* pv. *carotovora*, *X. campestris* pv. *vesicatoria*, and *C. michiganense* pv. *michiganense*. Antagonistic activity found for some of the combinations was associated with siderophore production, although antagonism could not always be related to the iron-chelating mechanism alone. The two best antagonists were tested in the greenhouse for control of bacterial speck of tomato caused by *P. syringae* pv. *tomato* in tomato seedlings. Both antagonists (unidentified *Pseudomonas* species) controlled the disease more efficiently than the copper treatments, but were not as effective as streptomycin at 1000 ppm.

Gitaitis (36) briefly examined the effect of *Erwinia herbicola*, a common corn epiphyte previously known to be antagonistic to other bacteria, and *Klebsiella pneumoniae*, a fast-growing bacterium, on the multiplication of *P. avenae*. Results obtained in vitro and in planta were inconclusive, although the sweet corn-*P. avenae* was suggested as being an interesting pathosystem for biological control studies.

CHAPTER THREE
DISTRIBUTION OF POPULATIONS OF EPIPHYTIC BACTERIA IN THE WHORLS OF SWEET CORN PLANTS

Introduction

Corn (Zea mays L.), as well as other upright grasses, has anatomical characteristics that make it a very efficient system for catching and retaining water. Rain, mist, dew, and guttation water run down the lamina, carrying with them leaf leachates, pollen, dust, and other particles from the atmosphere deposited on the surface. Microorganisms, especially bacteria, which are known to form epiphytic populations on leaves of most, if not all, plant species, are also carried into the whorl (32,42,54,55,75). Part of this fluid is collected in the whorl and in the cisterns formed by the collar and sheath of older leaves (75). According to Gitaitis (35), the sites of water collection correspond to the areas where symptoms of bacterial leaf blight and stalk rot (BLBSR), incited by Pseudomonas avenae, occur in corn plants. Therefore, the composition of this fluid is an important factor in determining the suitability of the leaf environment to P. avenae.

Studies on biological control of foliar diseases with bacteria generally involve measurement of initial populations of the antagonist at the infection unit or the ability of these populations to survive on plant surfaces (16,22,63,66,73,74,77,85,86,88). Measurements of populations of epiphytic bacteria have usually been made through the bulk-sampling procedure (42), with the exception of Crosse (21), who accounted for the high variability in the populations of *Pseudomonas syringae* pv. *morsprunorum* among individual cherry leaves. In 1982, Hirano et al. (42) examined the variability and distribution of epiphytic bacterial populations on individual leaves of various plant species and found that these populations follow a lognormal distribution, i.e., a distribution in which the logarithm of the original variate is distributed normally. It meant that estimation of population sizes based on bulked samples overestimates the population mean by a factor of approximately 1.15 σ^2. A lognormal distribution was also found for the total and specific bacterial populations in the rhizosphere of greenhouse and field plants of five species (65).

The objective of this study was to determine the variability and the distribution of populations of epiphytic bacteria in the fluid accumulated in the whorls of two cultivars of sweet corn, as related to the biological control of BLBSR of corn incited by *P. avenae*.

Materials and Methods

Sampling Procedures

The experimental plot consisted of 18 rows of sweet corn planted at the Horticultural Unit, University of Florida, Gainesville. Each three consecutive rows were planted on April 5, 1985, with either cultivar Merit or Silver Queen (Hastings Seed Co., Atlanta, GA), which are known to be susceptible and resistant to BLBSR, respectively (83). A resistant and a susceptible cultivar were used in order to determine whether different cultivars of the same species harbor distinct populations of epiphytic bacteria due to different compositions of leaf leachates or other factors (see Chapter Four). The distance between plants was approximately 20 cm in rows of 100 m. The rows were 0.8 m apart. Cultural practices recommended for the crop in Florida (81) were followed throughout the season.

Sampling was performed every five or six days, depending on the presence of free water in the whorl. Sampling began 30 days after planting, when the plants were at the eight-leaf stage, until silking started. A total of 24 plants per cultivar was sampled on each of the five sampling dates. Approximately 0.5 ml of fluid was withdrawn early in the morning from the whorl of eight randomly selected plants in the center row by using individually wrapped disposable transfer pipets (Fisher Scientific, Pittsburgh, PA). The plants were marked with colored tape and numbered, and the periodical collections were thereafter obtained from the same individuals. The pipets containing fluid from individual plants were sealed and transferred to the laboratory in a cooler. The samples were processed in the same day. Each sample was tenfold diluted five times in tubes with sterile tap water, and 0.05 ml aliquots of the three last dilutions were spread on Trypticase soy agar (TSA) (BBL Microbiology Systems, Cockeysville, MD) and on King's medium B (KMB) with a sterile glass rod. A bulk sample made by mixing the first dilution of all individual samples of a given treatment (for example, from cultivar Merit, at sampling date 1) was also diluted and plated. Bacterial colonies were counted after three days of incubation at 30 C. No fungal growth was observed for any of the dilutions which allowed colony counts (less than 300 colonies per plate).

For each cultivar and sampling date, the total population and the population of three groups of bacteria (fluorescent pseudomonads, *Erwinia herbicola*, and *Flavobacterium* sp.) were counted, for a total of 40 data sets. These three groups were chosen due to their prevalence on the plates and to the ease of separation based on their colony characteristics. Their identity was confirmed through quick tests such as shape of the cells, motility, Gram stain, anaerobic growth, and production of catalase and oxidase. Colonies of fluorescent pseudomonads were counted on KMB, whereas the other two groups and the total number of colonies were counted on TSA and KMB. In order to prevent an underestimation of the number of colonies due to presumable in vitro antibiosis, counts were made on plates with less than 50 colonies whenever the total count did not decrease proportionally with the dilution. Counts on duplicate plates accounted for the lack of accuracy of utilizing a low number of colonies for estimation of populations, which were expressed in colony-forming units per milliliter (cfu/ml).

Data Analysis

Two procedures were used in order to determine if the normal distribution can be used to describe the distribution of bacterial populations in individual whorls. First, in the graphical procedure, a normal probability plot of the residuals was constructed according to Montgomery (68). The residuals were found by subtracting the data set average from each observation of that data set. A normal probability plot is a graph of the cumulative distribution of the residuals on normal probability paper, that is, a graph paper with the coordinate scaled so that the cumulative normal distribution plots as a straight line. The normal probability plots were constructed by arranging the residuals in increasing order and the Kth value of these ordered residuals was plotted against the cumulative probability point $P_k=(K-1/2)/N$, where N is the number of observations. If the underlying error distribution is normal, this plot will resemble a straight line. In order to facilitate the visualization of the results, the residuals on the graph were substituted by their respective original values, i.e., the bacterial populations or the logarithm of the bacterial populations.

In the second method, the Shapiro-Wilk (80) test for normality was performed according to a univariate procedure provided by SAS (Statistical Analysis System, Release 5.08, SAS Institute Inc., Cary, NC). In this procedure, the test statistic W is interpreted as the correlation between the spacing of the ordered data and that of the ordered expected values (80). The W value will approach one if the observations on the data set are normally distributed; as the data depart from normality, W will become smaller than one. The P value describes the confidence level with which one can reject the null hypothesis of normality.

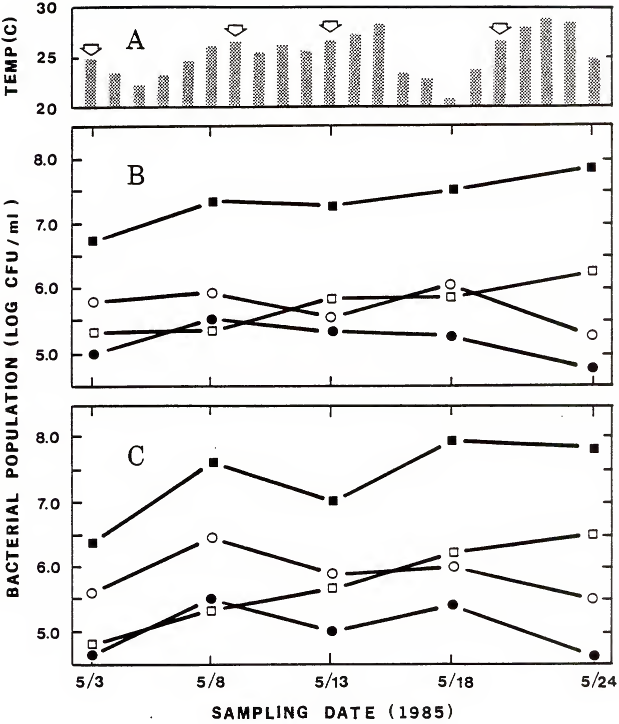
The tests for normality were performed for the 15 data sets which had more than 15 observations. The data sets with 10 or more missing values, due to numbers of bacteria below the detection level, were not considered large enough for the study.

Results

Variability of Bacterial Populations in Individual Whorls

The number of epiphytic bacteria from both sweet corn cultivars was quite variable from plant to plant and in the same plant at different sampling times. Numbers of total epiphytic bacteria were less variable than any of the other three groups studied. They varied by a factor of less than 100 for each sampling date and for each cultivar. For any of the groups of bacteria, this factor was often above 10^4, and ranged from undetectable to numbers close to the total population. When the same plants were sampled at different times, there was a tendency of some individuals to preserve specific groups of bacteria. For example, _Flavobacterium_ sp., a commonly isolated epiphyte, could not be recovered from a group of nine neighboring plants on any of the five sampling dates. Plants that had one group of bacteria in the first sampling date tended to harbor this group in the subsequent samplings. The variation over time of the average of total bacterial populations and of populations of specific groups is shown in Fig. 3-1. The number of colonies from the bulked samples obtained by mixing the first dilution of individual samples for each time and variety indicated that the undetectable numbers of bacteria were closer to the detection level than to zero. Bulking

Fig. 3-1. Populations of total bacteria (■), fluorescent pseudonomads (●), *Erwinia herbicola* (o), and *Flavobacterium* sp. (□) isolated from sweet corn cultivars Merit (B) and Silver Queen (C) on five sampling dates. Numbers represented are the average of the logarithm of the populations on 18 to 24 plants of each cultivar on each sampling date. Samples for which any of the three groups of bacteria were not detected were assigned the value of the detection limit for that date. The daily average temperature for the period is represented in A. Arrows indicate when rain occurred.


TEMP(C)
30
25
20
A
B
C
BACTERIAL POPULATION (LOG CFU/ml)
8.0
7.0
6.0
5.0
5/3
5/8
5/13
5/18
5/24
SAMPLING DATE (1985)

caused an overestimation of bacterial numbers in individual whorls by a factor of 1.71 σ^2.

Distribution of Bacterial Populations

The distribution of bacterial populations estimated in individual whorls in cfu/ml was consistently found to be positively skewed when plotted on a histogram, as shown for one of the data sets (Fig. 3-2A). It means that a high number of individual whorls harbor a relatively low number of bacteria, while fewer whorls have unusually high populations. This pattern was more evident for the selected individual groups of bacteria, as compared with total bacteria, which tended to be quantitatively less variable. When the original population values were transformed to $\log_{10}$ cfu/ml, a more symmetrical distribution was obtained (Fig. 3-2C). When a normal probability plot of the residuals was constructed on a normal probability paper against the cumulative probability point, a substantial curvature for the data was evident with the nontransformed values, whereas the log-transformed values approximated a straight line (Figs. 3-2B, 3-3, 3-4). Therefore, the data represented in Figs. 3-2, 3-3, and 3-4 for total and fluorescent bacteria, and for *Erwinia herbicola*, respectively, strongly support the notion that the populations of bacteria in the whorl of corn plants in the field are better described by the lognormal than by the normal distribution.

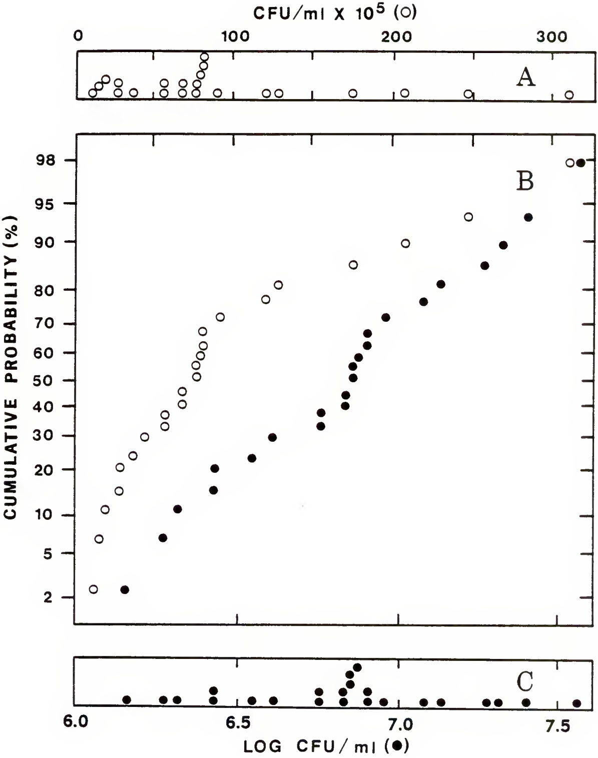


Fig. 3-2. Cumulative probability plots of total bacterial populations in the fluid accumulated in the whorl of plants and sweet corn cv. Merit in the field (B). The population sizes are indicated in cfu/ml (A) or log cfu/ml (C).

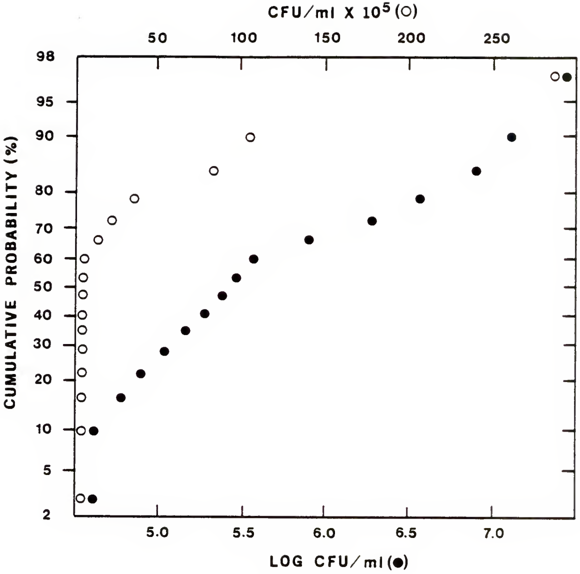


Fig. 3-3. Cumulative probability plots of populations of fluorescent pseudomonads in the fluid accumulated in the whorl of sweet corn cv. Merit in the field.

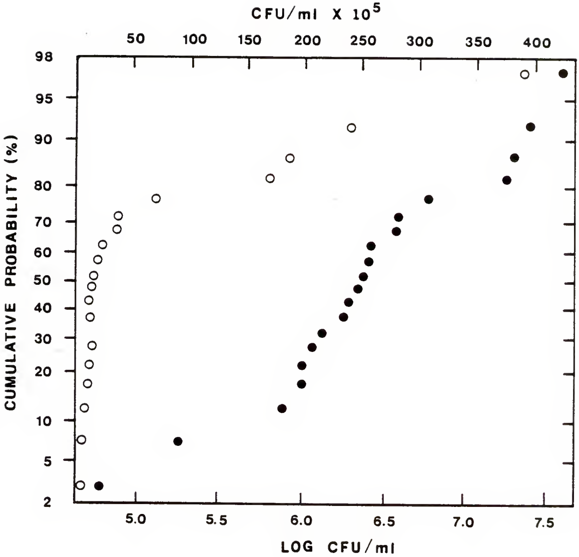


Fig. 3-4. Cumulative probability plots of populations of *Erwinia herbicola* in the fluid accumulated in the whorl of plants of sweet corn cv. Merit in the field.

The Shapiro-Wilk test indicated that the null hypothesis (which indicates that a normal distribution describes a set of observations) was not rejected for 10 of the 15 data sets when the population numbers were logarithm transformed. When the nontransformed values were used, only 4 of 15 data sets were considered to be normally distributed (Table 3-1).

Discussion

The population mean and the median are described by the same value when the distribution is normal. The lognormal distribution is positively skewed, causing the mean to be greater than the median. As illustrated by Hirano et al. (42) for ice nucleation-active bacteria, which were found to be lognormally distributed, a disproportionate number of cells from the largest populations contribute to the arithmetic mean. Therefore, there is an overestimation of the mean if samples are bulked, since it involves an arithmetic averaging of the individual bacterial populations. This phenomenon was also observed in this study when the numbers obtained by the dilution of bulked samples were compared with the mean of the logarithm of populations in individual samples. Bulking caused an overestimation of bacterial numbers by a factor of 1.71 σ^2, as compared with the factor of 1.15 σ^2 found by Hirano et al. (42) for leaf-surface bacteria. Besides depending on the variance, the

Table 3-1. Test for normality of 15 data sets of bacterial populations from the whorls of sweet corn cultivars Merit (M) and Silver Queen (SQ).

Data Set	Sample Size	cfu/ml		Log cfu/ml	
		W	P	W	P
Total M1	23	.80	<.01	.97	.70
Total M2	24	.60	<.01	.91	.05
Total M3	23	.63	<.01	.97	.66
Total M4	23	.77	<.01	.93	.16
Total M5	24	.93	.17	.91	.04
Total SQ1	16	.91	.13	.93	.34
Total SQ2	22	.96	.57	.86	<.01
Total SQ3	19	.71	<.01	.95	.47
Total SQ4	18	.95	.47	.90	.06
Total SQ5	23	.85	<.01	.95	.30
E. herbicola 1	20	.89	.04	.87	.01
E. herbicola 2	20	.62	<.01	.94	.33
Flavobacterium	15	.79	<.01	.87	.05
Fluorescent 1	16	.58	<.01	.89	.07
Fluorescent 2	16	.53	<.01	.91	.20

W and P values were calculated by the Shapiro-Wilk test for normality performed according to a univariate procedure provided by SAS. W values approaching one indicate normality. P values describe the confidence level with which one can reject the null hypothesis of normality.

Numerals in the data set indicate sampling date.

estimation of lognormally distributed populations based on bulked samples depend on the sample size (80).

The variation in the density of bacterial populations among plant whorls found in this study, especially when the selected groups of bacteria were considered, indicates that the population of a biocontrol agent on an individual plant, rather than the mean population among a set of plants, will influence the amount of disease on that plant. The same trend has been observed for leaf-surface (42) and rhizosphere (65) bacteria on several plant species. The whorl is different from unfurled leaves in that it harbors larger populations (at least 10 times more) of bacteria. The whorl is constantly moist and protected from ultraviolet light, which is deleterious to mainly nonpigmented bacteria. Another distinct feature of the furled in relation to unfurled leaf surfaces is that _P. avenae_ infects almost exclusively furled leaves. Because some plants tended to preserve specific groups of bacteria over time, I speculate that the predominance of a given species in a plant is mostly a chance factor. Whenever a good colonizer gets to its niche in a young plant, it will take over, if the environmental conditions are favorable, and will inhibit the growth of other colonizers.

The cumulative probability plots (Figs. 3-2, 3-3, 3-4) imply that, if a threshold population of a biocontrol agent against _P. avenae_ is determined, the frequency with which this threshold population is met or exceeded (through application or through multiplication and/or survival in the field) on individual plants may be utilized to predict the intensity of BLBSR of corn. It means also that sample collection for isolation of biocontrol agents in the whorls should be done on an individual plant basis rather than by bulking samples from an entire field or even sections of a field. The uniqueness of each whorl as an ecosystem suggests that the isolation of biocontrol agents should be aimed towards plants that do not show symptoms. It has been observed for the *P. avenae*-corn pathosystem that scattered plants in the field are symptomless, while the majority of the plants might be covered with lesions. This might be mainly due to developmental variation (like time of whorl formation), to structural conformation of the whorl in which water is not collected and, therefore, disease is not favored, or to the presence of naturally occurring biocontrol agent(s) effective against *P. avenae*. This last factor is in accordance with the assertion of Baker and Cook that biological control occurs at all times in nature (7).

CHAPTER FOUR
SCREENING OF EPIPHYTIC BACTERIA FROM CORN FOR ANTAGONISM TO *Pseudomonas avenae*

Introduction

Bacteria are known to form epiphytic populations on leaves of most, if not all, plant species. Many of these bacteria produce metabolites which are inhibitory to other microorganisms on the phylloplane. The literature on bacterial antagonists reported to suppress bacterial diseases on plants has been reviewed in Chapter Two.

Because antibiosis is a commonly assumed mechanism in biological control, most of the reports on suppression of bacterial diseases by epiphytic bacteria include in vitro tests for antagonism. The test consists basically of application of the presumed antagonist to a solid medium in a petri plate, allowing it to grow for some time, and then applying the target bacterium. The production of diffusible antibiotic substances by the first organism prevents the growth of the target organism, thus forming a zone of inhibition between the two colonies. Often, in vitro tests are used to explain or to suggest the kind of mechanism responsible for disease control observed in planta in the field or in the greenhouse (40,59,62,63,66,77,78,85,88).

In general, the absence of an antagonistic effect in vitro has led to the conclusion that mechanisms other than antibiosis are involved in antagonism. Even though this might be true in some cases, the production of secondary metabolites by microorganisms is influenced by culture conditions, and agar plates and leaf surfaces are fundamentally different (24,46).

Bacterial leaf blight and stalk rot (BLBSR), caused by *Pseudomonas avenae*, develops in parts of the corn plant where water accumulates, especially the whorls (36). This pathosystem was, therefore, ideal for studying the effect of metabolites produced by epiphytic bacteria present in the whorl water on disease development.

Teliz-Ortiz and Burkholder (85) used potato dextrose agar (PDA) to test for antibiotic production by *Pseudomonas fluorescens* against many plant pathogenic bacteria. No inhibition zones were observed when nutrient agar (NA) was used as the test assay medium, probably because NA, somehow, neutralizes the antibiotic principle produced by *P. fluorescens* (85). Riggle and Klos (74) used a medium containing sugar concentrations comparable to those in pear nectar to test the antagonistic effect of *Erwinia herbicola* against *E. amylovora*. It was found that *E. herbicola* consumed all organic nitrogen in the medium, besides reducing its pH to levels inhibitory to *E. amylovora* (74).

Ho and Ko (44) reported that agar in a test medium reduced the inhibitory effects of certain antibiotics and suggested Sea Kem agarose as a solidifying agent in the assay medium. I have not observed differences in the diameter of inhibition zones produced by six antagonists to two cultures of _P. avenae_ on Trypticase soy medium solidified with either Difco bacto agar or Sea Kem agarose (unpublished data).

Antagonism in vitro due to iron competition by production of siderophores, bacteriocin-like compounds, or other types of antibiotics was examined by Lindow (61) on KMB, NA, TSA, Luria agar, and mineral salts. Only 58% of 88 bacterial cultures which reduced epiphytic growth of _Pseudomonas syringae_ on plant surfaces produced compounds inhibitory to it on any of the media tested. The production of iron-regulated antibiotics, such as siderophores, was indicated by an elimination of the antibiotic effect when ferric chloride was added to the medium. The production of bacteriocin-like substances was indicated by suppression of antibiosis when trypsin was supplied to the medium before application of _P. syringae_ (61).

It has been reported that different varieties of the same plant species may harbor distinct epiphytic microbial populations and that these differences could be a consequence of different compositions of the plant leachates (38). For example, Crosse (21) and Daub and Hagedorn (25) found that the number of epiphytic *Pseudomonas syringae* cells was higher on surfaces of susceptible than on resistant bean varieties. Wheat isolines differing only in their resistance levels to *Cochliobolus sativus* root rot had vastly different root microflora. About 20% of bacteria isolated from the rhizosphere of a resistant variety exhibited antibiosis in vitro against *C. sativus*, compared to none from the susceptible variety (3). Naqvi and Chauham, cited by Suslow (84), reported that pepper (*Capsicum annuum*) varieties resistant to *Fusarium oxysporum* produced root exudates which inhibited germination of spores of *F. oxysporum*, whereas exudates from susceptible varieties enhanced it. Furthermore, resistant varieties had higher populations of potential antagonists such as *Trichoderma viride* and *Aspergillus sydowi* (84). According to El-Zik et al. (28), differences in the microflora of cotton seeds and roots are apparently due to a genetic control of the quality and quantity of exudates. The altered exudates are claimed to be nutritionally unfavorable to pathogens and favorable to microorganisms which are antagonistic to them (28). Azad et al. (6) found that populations of bacteria that exhibited antagonism in vitro to *Verticillium dahliae* were higher in the rhizosphere of resistant than in susceptible potato genotypes.

The lack of correlation between microbial antagonism in vitro and effectiveness of disease control in the field has become evident in biological control studies (1,61,89). Examples of good antagonists in vitro failing to control disease in the field and, conversely, efficient antagonists in the field that do not form zones of inhibition on petri dishes, are found in Andrews (1) and Andrews et al. (2).

Tests for antagonism in plants growing under controlled conditions was considered by Andrews (1) as the key assay for biological control agents. Any candidate that does not perform well under close to optimal environmental conditions and relatively free of competitors can be discarded. Therefore, reproducible, large-scale experiments are needed but, often, they are difficult to accomplish.

For the sweet corn-*P. avenae* pathosystem, I have observed that only approximately 50% of the plants of the susceptible cultivar Merit developed visible lesions when inoculated in the whorl with 10^7 cfu/ml of *P. avenae*. As a result, a large number of plants must be inoculated in order to detect significant differences among treatments for the control of the disease. Hartman and Kelman (41) have found that development of symptoms of bacterial stalk and top rot of corn caused by *Erwinia chrysanthemi* pv. *zeae* was very erratic when plants were inoculated in the whorl. The addition of 0.7% of Tween 40 in the inoculum, however, resulted in a consistent rotting and collapsing of plants in the greenhouse.

Strains of a pathogen may differ in their susceptibility to a given antagonist (2). Therefore, various strains of a pathogen should be routinely tested to avoid an eventual failure in disease control due to the prevalence of a strain which is not inhibited by the biocontrol agent.

The applications of infectivity titration to estimate pathogen virulence and plant host resistance have recently been reviewed by Ercolani (29). The most important feature of this technique is that it allows the detection of relatively small differences in resistance among plant varieties or differences in virulence among strains of a pathogen. Often, these differences are not detectable with standard screening procedures, since graded inoculum doses overcome some of the disadvantages of a single, severe inoculation.

With the foregoing in view, the objectives of this study were (1) to examine epiphytic bacteria isolated from the leaves and whorls of corn plants from different locations in Florida for their ability to produce diffusible substances antibiotic to _P. avenae_ in vitro; (2) to verify if the resistant cultivar Silver Queen harbors higher numbers of antagonistic fluorescent pseudomonads, as compared with the susceptible cultivar Merit; and (3) to screen these bacterial cultures for antagonism to _P. avenae_ in planta in the greenhouse.

Materials and Methods

Isolation of Epiphytic Bacteria

Fluid accumulated during the night in the top funnel of furled leaves was collected in the morning (before 8:00 a.m.) from plants in the vegetative stage (30 cm tall to tasseling) of different varieties and inbred lines of field and sweet corn in Florida. The samples were collected in 1983, 1984, and 1985 in Zellwood (AREC-Sanford Zellwood Farm), Belle Glade (Everglades Experimental Station), and Gainesville (Horticultural Unit and Agronomy experimental plots). Bacterial diseases were absent in all locations at the time of collection, except for Belle Glade, where BLBSR severity in specific plots ranged from slight to severe, depending on the geotype and on the developmental stage of the plants. In these plots, samples were collected from symptomless plants. Otherwise, sampling was done at random, following a W pattern. Ten to 20 plants were sampled per hectare, depending on the size of the field. Approximately 0.5 ml of fluid was withdrawn from the top funnel of individual plants with individually wrapped disposable pipets which were then sealed, placed in a cooler, and transferred to the laboratory. The samples were processed in the same day. Six tenfold dilutions on sterile tap water were performed in test tubes, and 0.05 ml of the last four dilutions were spread with a sterile glass rod on duplicate plates of Trypticase soy agar (TSA) or

King's medium B (KMB). After two days of incubation at 30 C, individual colonies of the two to four most prevalent species, based on colony appearance, were streaked to TSA plates to assure purity and stored in sterile tap water in 2-ml vials at room temperature (22-24 C). The dilution plates which yielded 20-100 colonies per plate were then sprayed for one second with approximately 5×10^6 cfu/ml of culture 83-1 of *Pseudomonas avenae* grown on NA for 24 hours at 30 C and suspended in sterile tap water. A 3-oz chromist spray unit (Gelman Instrument Company, Ann Arbor, Michigan) was used to spray the plates. The plates were incubated for 24 hours at 30 C and examined for zones of inhibition. Colonies which formed zones of inhibition were streaked twice on TSA plates and transferred to vials of sterile tap water for storage. This procedure allowed the isolation of the most prevalent bacteria on individual plants which presumably were, therefore, good colonizers and could compete for sites or nutrients with the pathogen. The procedure allowed also the isolation of bacteria that were present not in as high numbers on the plants, but produced any diffusible compound inhibitory to *P. avenae*. Whenever the same colony type from samples from the same area appeared in many plates, only four to six of these cultures were stored. Because pseudomonads represent the major group of nondifferentiating microorganisms producing antibiotics (57), sixty cultures of fluorescent bacteria were isolated from sweet corn cultivars Merit and Silver Queen in a 21-day period at the Horticultural Unit. A total of 179 cultures were isolated using these procedures.

Antagonism In Vitro

A wettable powder formulation of *Bacillus* sp. (ABG 4000) previously known to be antagonistic in vitro to *P. avenae* (A. J. Crovetti, personal communication to Dr. R. E. Stall), was provided by Abbott Laboratories, North Chicago, Illinois, and was included in the tests. Droplets of a turbid suspension of each of the 179 bacterial cultures (about 10^8 cfu/ml) were spotted with a 1 mm loop on a TSA plate. Three equidistant droplets were placed on each of three plates. The spotted plates were incubated at 30 C for 48 hours and then sprayed with approximately 5×10^6 cfu/ml of *P. avenae* with a chromist sprayer as already described. The plates were then reincubated at 30 C for 24 hours. The diameters of the zones of inhibition were recorded (diameter of zone of inhibition = total diameter of zone - diameter of the colony of the antagonist). Unless otherwise specified, only zones with diameter larger or equal to 4 and 10 mm for TSA and KMB, respectively, were considered.

Whether or not different cultures of *P. avenae* were equally inhibited by diffusible substances from a given epiphyte was determined with a factorial experiment with three replications. Nine antagonists known to produce zones of inhibition on TSA to strain 83-1 of *P. avenae* were tested against six cultures of the pathogen. The application of the antagonists and the pathogen to the plates and the data collection were performed as previously described.

Antagonists in Resistant and Susceptible Cultivars

Plants of the cultivars Merit and Silver Queen, susceptible and resistant to BLBSR, respectively, were sampled five times every five or six days. The experimental design and the sampling procedures are described in Chapter Three. Thirty fluorescent pseudomonads were randomly selected from dilution plates of fluids from the whorls of plants of each cultivar. The production of diffusible substances antagonistic to *P. avenae* was tested in plates of TSA and KMB. The procedures for the in vitro test are as already described.

Preliminary Screening In Planta in the Greenhouse

Sweet corn plants of the cultivar Merit (Hastings Seed Co., Atlanta, GA) were grown in a greenhouse (26-40 C) from seeds in 10-cm plastic pots containing steam-sterilized Metro-mix 300 Growing Medium (Grace Horticultural and Agricultural Products, Cambridge, MA). The plants were kept on a greenhouse bench (25-38 C) in a randomized block design with two blocks. Each treatment consisted of a row of eight pots with three plants each. The antagonistic bacteria isolated from corn plants, referred to previously, were grown in TSB at 30 C, centrifuged at 2000 rpm for 10 minutes, and resuspended in sterile tap water. The suspension was adjusted to contain approximately 10^8 cfu/ml through optical density measurement in a spectrophotometer at 600 nm. For each antagonist, 120 µl of the bacterial suspension was pipeted into the whorl of 10-12 day-old seedlings, which had the fourth leaf half to two-thirds unfurled. Care was taken not to disturb the plants, so the liquid would remain in the whorl. Plants treated with 120 µl sterile tap water were used as controls.

Inoculation with _P. avenae_ consisted of pipeting 120 µl of $5x10^6$ cfu/ml of bacterial suspension with or without 0.01% Tween 20 into the whorl, 12 or 24 hours after the application of the putative antagonists to the seedlings. The culture 83-1 of _P. avenae_ used in the inoculations was isolated from a diseased corn plant from Belle Glade, Florida. The pathogen was grown for 24 hr in nutrient broth at 30 C, centrifuged for 10 minutes at 2000 rpm, resuspended in sterile tap water, and the concentration of bacteria determined through optical density in a previously calibrated spectrophotometer at 600 nm. A 200-µl automatic pipet (Pipetman, Rainin Instrument Co., Inc., Woburn, MA) was used for the application of both the epiphytes and the pathogen into the whorls.

The epiphyte bacterial cultures were screened for antagonism in eight experiments, four without and four with

Tween 20 added to the suspension of the pathogen. Disease was assessed four to six days after inoculation. When Tween 20 was not added, disease incidence was recorded, i.e., the percentage of plants with BLBSR symptoms, regardless of the amount of disease on the leaves. When Tween 20 was added, disease severity was recorded by using a scale from 0 to 5, with 0 representing no symptoms and 5 representing dying or dead plants. The intermediate values were based on the percentage of the surface of the fourth leaf covered with water-soaked or necrotic lesions. The mean value for each antagonist in each replication was transformed to represent the percentage of disease in relation to the water control. For example, a value of 0.50 would mean that the seedlings treated with a given antagonist developed 50% of the amount of disease developed in the water control. This transformation enabled the selection of the best antagonists in individual experiments, regardless the amount of disease in the control plants in that experiment. Analysis of variance and Duncan's multiple range test were performed through a SAS program for each data set.

Effect of Tween 20 on BLBSR Development

The plants and the bacterial suspensions for this experiment were prepared as for the screening procedure. The treatments consisted of adding 0.01, 0.05, 0.10, 0.50, or 1.00% of Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma Chemical Co., St. Louis, MO) to a suspension containing approximately 5x10^6 cfu/ml of *P. avenae*. Control treatments without Tween 20 and without *P. avenae* were included. Inoculations were made by pipeting 120 μl of the suspensions into the whorl of 12-day old seedlings. The inoculated plants were kept in a greenhouse bench (26-40 C) in a randomized block design with five blocks. Each treatment consisted of eight pots with three plants each. Seedlings that did not retain the inoculum in the whorls because of developmentally related leaf structure were eliminated at the time of inoculation. Disease incidence (percent of plants with any amount of disease) and disease severity (scale 0 to 5, as described previously) were recorded five days after inoculation.

Final Screening

The ten most promising antagonists detected in the preliminary greenhouse screening for disease control were further tested, along with the five best antagonists detected in petri dish tests, for control of BLBSR on seedlings of sweet corn (cv. Merit) in the greenhouse. They were also retested for antagonism in vitro against *P. avenae* on TSA medium as before. The conditions for the experiment in the greenhouse were similar to those for the preliminary screening, except that a randomized block design with three replications of eight pots each, three plants per pot, was used. Tween 20 at 0.01% was added to the inoculum of *P. avenae* immediately prior to inoculation. Inoculum was applied 12 hours after the application of the antagonist to plants. Streptomycin at 100 ppm was applied as a control in the same amount and at the same time of application of the antagonists. A treatment in which water was applied instead of an antagonist was used as a control. Disease severity was assessed five days after inoculation, with the previously described scale of 0-5 ratings.

Identification of Best Antagonists

Antagonists F-11 and F-24 were isolated from the whorl of different, disease-free sweet corn plants of cultivar Merit grown at the Horticultural Unit in the summer of 1985. The tests for the identification of the two antagonists were performed simultaneously with two known cultures of each *Pseudomonas putida*, *P. fluorescens*, and *P. aeruginosa*, which were the species that most resembled the unknown bacteria. The standard medium for testing the utilization of carbon and nitrogen sources by fluorescent pseudomonads was that proposed by Misaghi and Grogan (67). The selection of tests and of substrates used to differentiate the species of fluorescent pseudomonads and biovars of *P. fluorescens* were obtained in Gilardi (34), Misaghi and Grogan (67), Palleroni (72), and Sands et al. (76).

Antagonist U-46 was isolated at Sanford, Florida, and was identified based on the following characteristics: cell shape and motility, gram stain, production of endospores, production of catalase, and oxygen requirement for growth (33).

Antagonism of F-11 to Different Cultures of *P. avenae*

Cultures 73-20, 74-02, 78-05, and 78-07 of *P. avenae* were obtained from Dr. R. E. Stall's collection, University of Florida, Gainesville. Cultures 83-1 and 84-1 were isolated in 1983 and 1984, respectively, from corn leaves collected in Belle Glade, Florida, showing lesions typical of BLBSR. The experiment was conducted in a greenhouse. Preparation of plants and of inoculum and antagonist suspensions were as for previous experiments. Plants in one of two rows of seven pots with three plants each were treated with the antagonist in the late afternoon by pipeting into the whorl 120 µl of a suspension of approximately 10^8 cfu/ml of F-11. The two neighboring rows were then inoculated by pipeting 100 µl of suspension of $5x10^6$ cfu/ml of each culture of *P. avenae*. Each treatment was replicated three times in a randomized block design. Tween 20 at 0.01% was added to the bacterial suspensions prior to inoculation. Statistical analysis was performed through a Student's T test provided by EPISTAT Statistical Package for the IBM Personal Computer.

Infectivity Titrations for Quantifying Antagonism of F-11

Application of F-11 to plants was accomplished by pipeting 100 µl of a suspension containing approximately 10^8 cfu/ml of the antagonist. Antagonist F-11 was applied to half of the rows. Each row had seven pots and each pot had three plants. Inoculum of P. avenae was prepared by growing strain 83-1 in TSA for 48 hours at 30 C. The bacterial colonies were suspended in sterile tap water to reach $OD_{600}=0.1$, which corresponded to approximately 10^8 cfu/ml. Serial dilutions were then made to provide seven tenfold levels of inoculum ranging from 10^2 to 10^8 cfu/ml. Inoculation was done in the whorls 12 hours later with 120 µl of the suspension. Each inoculum level was applied to two rows, one treated and other not treated with the antagonist.

The experimental design was a randomized block design with three replications. Disease severity (0-5 scale as previously described) and disease incidence (percent plants infected) were assessed six days after inoculation. Linear regression of disease severity and incidence values (ordinate) vs. log dose of P. avenae (abscissa) was used to determine an ED_{50} value. The ED_{50} value is defined as the number of cfu/ml of the pathogen required to cause lesions in 50% of the plants (for disease incidence) or to induce a disease severity level correspondent to 50% of the maximum level obtained in the experiment.

Results

Antagonism In Vitro

Thirty-four of the 179 bacteria tested inhibited the growth of *P. avenae* on TSA plates. Inhibition was determined by the inhibition zones around the colonies of the antagonists (Fig. 4-1). Among the 34 antagonists, 29 belonged to the fluorescent pseudomonads, four were of the genus *Bacillus*, and one was of an unidentified genus.

There was an interaction between antagonist and culture when nine selected antagonists were tested for production of a zone of inhibition to six cultures of *P. avenae*. It could be noted, however, that the antagonists which produced the largest zones were effective against all the strains of the pathogen (Table 4-1). Only antagonist U-44 did not inhibit all cultures of *P. avenae*.

Number of Antagonists in Resistant and Susceptible Cultivars

From the 60 cultures of fluorescent pseudomonads tested, 35 produced zones of inhibition in vitro to *P. avenae* in KMB, 17 being isolated from cultivar Silver Queen and 18 from cultivar Merit. Twenty-four cultures produced zones on TSA, 11 from "Merit" and 13 from "Silver Queen." Sixteen cultures (eight from each cultivar) produced zones

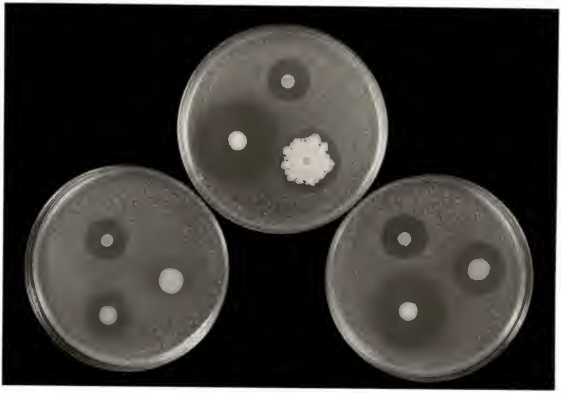


Fig. 4-1. Zone of inhibition to Pseudomonas avenae produced by different bacterial antagonists on Trypticase soy agar. The culture at the top of each plate is a Bacillus species (ABG 4000) used as a control. All others are epiphytic bacteria isolated from corn plants.

Table 4-1. Diameter (mm) of zones of inhibition produced in vitro by epiphytic bacteria antagonistic to cultures of _Pseudomonas avenae_.

Antagonist	Strain of _P. avenae_					
	73-02	74-02	78-05	78-07	83-01	84-02
U-132	25.7a	26.0a	26.0a	23.0a	25.0a	24.7a
U-133	24.3a	19.7b	24.3a	20.3b	22.3b	21.7b
ABG 4000	18.6b	10.0c	14.0b	10.3c	14.3c	14.0c
U-92	16.3c	10.7c	12.7b	10.3c	14.0c	12.3cd
U-23	19.3b	10.3c	10.7c	11.7c	12.0d	10.7d
U-51	15.3c	10.0c	12.7b	10.0c	13.7cd	12.0d
U-90	9.0d	2.7d	5.7d	6.3d	2.7g	1.3f
U-46	6.0e	2.7d	3.3e	3.0e	4.7f	2.3f
U-44	0.0f	0.0e	0.0f	0.0f	8.0e	7.7e

Numbers are average of three replications. Numbers followed by the same letter in each column are not significantly different according to Duncan's multiple range test (P=0.05). ABG 4000 is a _Bacillus_ species used as a control.

on both media. There were no statistically significant differences in the number of antagonists for the two cultivars in either of the culture media.

Preliminary Screening

Nine of the 11 best antagonists selected in the preliminary greenhouse screening were fluorescent pseudomonads (Table 4-2). The disease indexes were not statistically compared among themselves because they were obtained from different experiments. None of the cultures tested seemed to synergistically interact with *P. avenae* to increase disease development.

Effect of Tween 20 on BLBSR Development

Plants had no visible symptoms of phytotoxicity even when treated with the highest concentrations of Tween 20 without *P. avenae*. Development of BLBSR on sweet corn was noticeably enhanced when Tween 20 was added to the inoculum suspension (Fig. 4-2). When the two highest concentrations of Tween 20 were used, water soaking could be noticed in the stalks 48 hours after inoculation. Two days later, extensive stalk rot occurred, causing the death of the plants. For the three lowest concentrations of Tween 20 and for the control without it, symptoms developed especially on the leaves which were half unfurled at the time of inoculation. The stalk or the meristematic region was seldom affected. A lower incidence (approximately 60%) of disease was observed when plants were inoculated with a

Table 4-2. Control of bacterial leaf blight and stalk rot of corn in the greenhouse by bacterial antagonists (preliminary screening).

Antagonist[a]	Fluorescence[b]	Disease Index[c]
F-4	+	0.57
F-11	+	0.19
F-13	+	0.58
F-24	+	0.64
F-27	+	0.55
F-41	+	0.43
F-43	+	0.79
F-59	+	0.60
U-23	+	0.49
U-46	-	0.64
ABG 4000[d]	-	0.66
Control		1.00

[a] Best antagonists from 175 cultures tested.

[b] Fluorescence on King's medium B (KMB).

[c] Disease index is the amount of disease developed as compared with the water control value of 1.00 (100%). The indexes were not statistically compared because data were taken from different experiments.

[d] ABG 4000 is a Bacillus species used as a control.

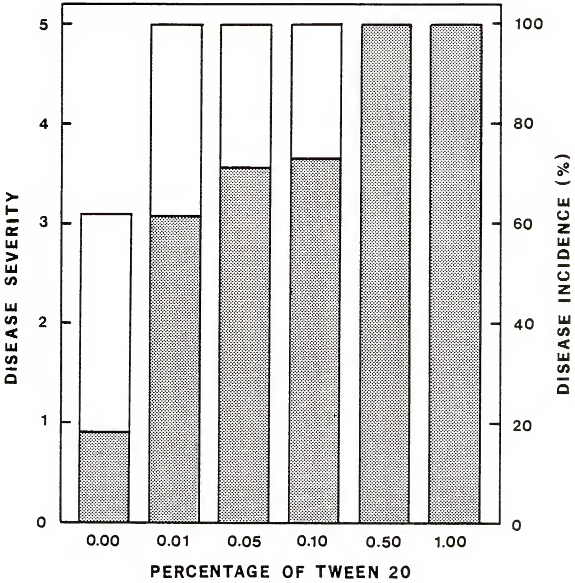


Fig. 4-2. Severity (shadowed bars) and incidence (clear bars) of bacterial leaf blight and stalk rot on sweet corn cv. Merit inoculated with suspensions of Pseudomonas avenae with different concentrations of Tween 20.

pathogen suspension without Tween 20. The average of the coefficients of variation for all the screening experiments were 7.5 and 34.5 when Tween 20 was used and not used, respectively.

Final Screening

Some antagonists that had performed well in the preliminary screening (such as F-4) were not as efficient in the final screening (Table 4-3). This difference might be attributed to variation in the conditions under which the experiments were conducted (especially environmental conditions). However, antagonist F-11 was outstanding in all the experiments in which it was included (F-11 was used as a control after it had been selected) (Fig. 4-3). No correlation was found by the standard method for calculating linear correlation (64) when the antagonists were ranked according to their ability to control BLBSR on sweet-corn plants and to produce a halo of inhibition against *P. avenae* in vitro (Table 4-3). In fact, a negative correlation (r=-0.71) was found; this was due mainly to the small zones of inhibition produced by F-11 and F-24 in vitro and to the inability of antagonist U-132 to control BLBSR in planta.

Identification of Antagonists

The most important characteristics of F-11 and F-24 were fluorescence on KMB (+); production of pyocyanin (-); hypersensitive reaction in tobacco and tomato leaves (-);

Table 4-3. Relationship between the efficiency of control of bacterial leaf blight and stalk rot on sweet corn cv. Merit in the greenhouse and the diameter of zones of inhibition produced by selected bacteria antagonistic to *Pseudomonas avenae*.

Antagonist	Test In Planta		Test In Vitro	
	Efficiency[a]	Rank	Inhib Zone[b]	Zone
F-11	.68a	1	.5	15
U-46	.55ab	2	6.0	9
F-24	.51b	3	1.0	14
STR[c]	.45bc	4	-	-
F-41	.33cd	5	3.0	11
F-13	.33cd	5	3.0	11
F-27	.33cd	5	9.0	6
F-43	.32cd	8	3.0	11
F-59	.28de	9	7.0	8
F-47	.23def	10	4.5	10
U-23	.21def	11	12.0	4
F-22	.19def	12	16.5	2
F-4	.13ef	13	9.5	7
W-1	.12efg	14	12.0	4
ABG 4000[d]	.07g	15	15.5	3
U-132	.03g	16	25.0	1
Control	.00g	-	-	-

[a] Efficiency based on percentage reduction of disease as compared with the water control.

[b] Inhibition zone in Trypticase soy agar (mm).

[c] STR = 100 ppm streptomycin.

[d] ABG 4000 is a *Bacillus* species used as a control.

Results given as average of three and two replicates for efficiency and inhibition zones, respectively. Numbers followed by the same letter are not significantly different according to Duncan's multiple range test ($P=0.05$).

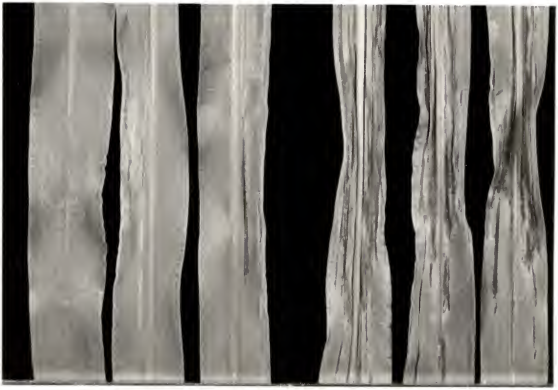


Fig. 4-3. Control of bacterial leaf blight and stalk rot on sweet corn cv. Merit in the greenhouse. The three leaves on the left were protected with culture F-11 of *Pseudomonas fluorescens* 12 hours before inoculation with *P. avenae*. The leaves on the right were not protected (water control).

growth at 42 C (-); growth at 41 C (+); potato rot (-); nitrate reduction (-); production of levan (-), oxidase (+), lipase (+), gelatinase (+), and lecithinase (-); utilization of acetamide (-), geraniol (-), mandelate (-), benzoylformate (-), adipate (-), trehalose (+), and alanine (+); and number of polar flagella (apparently 1). These results indicate that antagonists F-11 and F-24 most closely matched the description of *P. fluorescens* Migula (biovar V) and could not be separated from each other based on the tests performed or on colony appearance. Antagonist U-46 had rod-shaped, gram positive, motile cells with endospores. It grew only under aerobic conditions and produced catalase. These are characteristics of the genus *Bacillus*.

Antagonism of F-11 to Different Cultures of P. avenae

Disease suppression by antagonist F-11 in this experiment was unusually low for unknown reasons. Even so, F-11 significantly reduced BLBSR severity on sweet corn plants inoculated with four of six cultures of *P. avenae* (Fig. 4-4). No trend was observed in the efficacy of control in relation to the level of virulence of the pathogen.

Infectivity Titrations for Quantifying the Antagonism of F-11

There was a high correlation between log dose and disease intensity as indicated by the regressions from the infectivity titrations. A better correlation was found when disease severity, as compared to disease incidence, was assessed in both treatments, protected and not protected with antagonist F-11 (Table 4-4). Disease intensity was reduced by F-11 for all but the highest inoculum doses (Fig. 4-5). The ED_{50} values for the protected and nonprotected treatments were very distinct, regardless of the disease assessment method used (Table 4-4). The control factors for the antagonist, which were obtained by dividing the ED_{50} value of the protected by the ED_{50} value of the nonprotected treatments, were 1000 and 6025 when disease incidence and severity were assessed, respectively.

Discussion

Members of the fluorescent pseudomonads were the most prevalent among the bacterial epiphytes of corn that produced substances antagonistic to *P. avenae* in vitro. The same trend was observed when the same epiphytes were tested for their ability to control BLBSR on corn plants in the greenhouse. This indicates that the fluorescent pseudomonas should be given special attention in future surveys for biological control agents against BLBSR and perhaps other plant diseases. Leisinger and Margraff (57) have stated that pseudomonads represent the major group of nondifferentiating microorganisms producing antibiotics.

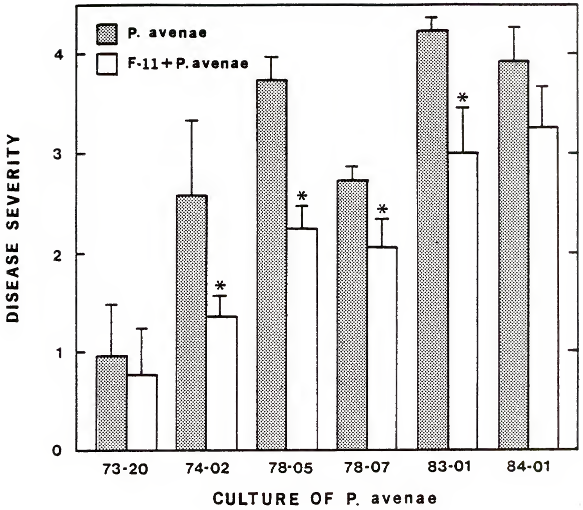


Fig. 4-4. Antagonistic effect of culture F-11 of *Pseudomonas fluorescens* to six cultures of *P. avenae* in the greenhouse. Numbers represented are average of 55-63 plants in three replications. Vertical lines represent the standard deviation from the means. * = Significantly lower at P=0.05 (T test).

Table 4-4. Infectivity titrations for quantifying the control of bacterial leaf blight and stalk rot on sweet corn cv. Merit with culture F-11 of *Pseudomonas fluorescens*.

Treatment[a,b]	Assessment	Regression Equation	r[c]	ED_{50}[d]	Log CF[e]
PA	Severity	Y=0.43x+0.18	0.99	$1.4x10^3$	3.78
PA + F-11	Severity	Y=0.26x-0.26	0.96	$8.3x10^6$	
PA	Incidence	Y=12.11x+12.37	0.94	$1.3x10^3$	3.00
PA + F-11	Incidence	Y=10.25x-12.57	0.91	$1.3x10^6$	

[a] PA = Strain 83-1 of *Pseudomonas avenae*.

[b] PA + F-11 = Inoculation with PA, 12 hours after application of antagonist F-11.

[c] r = Regression coefficient.

[d] ED_{50} = Inoculum dose inducing 50% disease incidence or 50% of the maximum value of disease severity in the test.

[e] CF = Control factor, obtained by dividing the ED_{50} values in treatments protected and not protected with *Pseudomonas fluorescens* F-11.

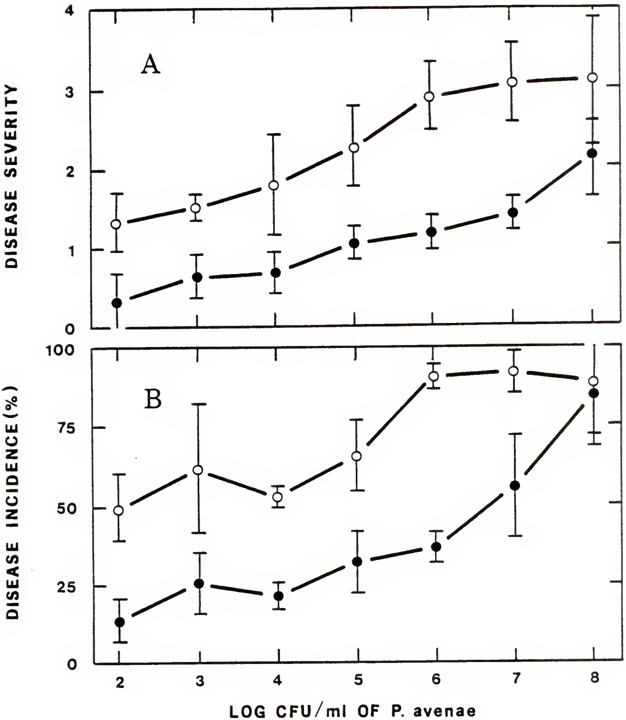


Fig. 4-5. Infectivity titration of Pseudomonas avenae on sweet corn cv. Merit protected (●) and not protected (○) with culture F-11 of P. fluorescens 12 hours before inoculation. Numbers represented are average of 54-63 plants in three replications. Vertical lines indicate the standard deviation from the means.

The study comparing antibiotic production on TSA plates and ability to control BLBSR on plants in the greenhouse by a selected group of antagonists indicated that there was no correlation between microbial antagonism in vitro and effectiveness of disease control in corn plants. This lack of correlation has previously been reported for bacteria colonizing the leaf surface of corn and other plants (1,61,89). The main implication of these results is that good biological control agents might be discarded if the agar plate assay is used as the sole criterion in an initial screening step, as in the scheme proposed by Blakeman (10).

Antagonists which are ineffective in planta but which form zones of inhibition in petri plates may be important, nevertheless, as material for the elucidation of mechanisms involved in disease control by successful antagonists under greenhouse or field conditions. They may also be of significance in reducing diseases other than that being studied. Furthermore, if disease control depends both on survival of the antagonist in the field and on antibiotic production, they can be useful in genetic engineering, which can provide means to increase fitness or to promote antibiosis in bacteria through gene transfer (58).

The interaction between antagonists and strains of _P. avenae_ observed in vitro indicates that a biological control agent might need to be tested for effectiveness against a representative collection of cultures of the pathogen. However, this statement can be conclusive only if the interaction is also observed in planta.

Merit and Silver Queen, sweet-corn cultivars susceptible and resistant to BLBSR, respectively, harbored identical numbers of epiphytic fluorescent pseudomonads which produce diffusible substances antagonistic to *P. avenae* in vitro. The number of in planta antagonists obtained from these two cultivars was also identical and indicates that the survey for fluorescent pseudomonads antagonistic to *P. avenae* could be made in any corn cultivar, regardless the level of resistance to BLBSR it carries.

The phenomenon of disease escape observed when corn plants are inoculated in the whorl with a water suspension of *P. avenae* cells was observed throughout the preliminary screening of antagonists. Even at concentrations as high as $5x10^7$ cfu/ml of the pathogen, disease incidence was seldom above 60%. This implies a need of a large number of inoculated plants for the detection of statistically significant differences. Concentrations of Tween 20 between 0.01 and 0.10 were considered adequate for screening purposes, since their application resulted in 100% disease incidence and intermediate levels of disease severity, which would allow the detection of smaller reductions of disease intensity. The effect of Tween 20 is to reduce the surface tension and facilitate the movement of inoculum in the spaces between the leaf blades in the whorl (36). It has not been determined how Tween 20 affects antagonism. However, I speculate that it might increase the plant protection by allowing the antagonist to be better distributed in the infection court.

The final screening of epiphytic bacteria for antagonism to P. avenae in corn plants in the greenhouse indicated that the treatment with antagonists F-11, U-46, and F-24 reduced disease in more than 50% in comparison with the untreated control. They also had at least 5% less disease than the treatment with streptomycin at 100 ppm.

Protection has been reported to be very erratic in most attempts to control disease through application of microbial antagonists, probably because the efficacy of the biocontrol agent is often largely affected by environmental conditions (20). This was not true for F-11, which was the most efficient antagonist in five tests in planta in the greenhouse (F-11 was used as a control since it has been found to be a potentially important biocontrol agent).

Strains of a pathogen may differ in their susceptibility to an antagonist; therefore, various cultures or mixtures of the pathogen should be tested before one attempts to control the disease in the field. Even under favorable disease conditions, antagonist F-11 significantly reduced the severity of BLBSR induced by four of six strains of P. avenae. It indicates that the protection given by F-11 is not restricted to strain 83-1 of *P. avenae*, against which it had been initially tested.

As for host resistance and pathogen virulence, small differences in disease suppression by biocontrol agents are usually masked by a single, severe inoculation. This phenomenon was observed in this study; no significant differences were found between treatments in which plants were protected and not protected with antagonist F-11 when plants were inoculated with a suspension of 10^8 cfu/ml of *P. avenae*. Therefore, infectivity titration can be a useful technique to detect small effects from the application of microbial antagonists as well as to compare the effectiveness of different antagonists in controlling disease.

Higher coefficients of correlation between inoculum dose and disease intensity were obtained when disease severity was assessed, indicating that the disease severity is a better criterion for assessing BLBSR of corn in greenhouse experiments. Based on the ED_{50} values, disease control by antagonist F-11 was equivalent to reducing the inoculum dose at least 1000 times. Indeed, protected plants inoculated with higher inoculum doses had symptoms similar to nonprotected plants inoculated with lower inoculum doses.

CHAPTER FIVE

BIOLOGICAL CONTROL OF BACTERIAL LEAF BLIGHT AND STALK ROT OF CORN IN THE FIELD WITH BACTERIAL ANTAGONISTS

Introduction

Saprophytic bacteria on leaf surfaces sometimes interact with plant pathogenic species resulting in a decrease in the amount of disease that develops (see Chapter Two for examples). It was shown in the previous chapter that some epiphytic bacteria control bacterial leaf blight and stalk rot of corn, incited by Pseudomonas avenae, under greenhouse conditions. The definitive test of a microorganism for its potential as a biocontrol agent includes, however, its antagonistic activity per se and its survival ability on the phylloplane (1). Failure of disease control in the field by an antagonist which was found effective in greenhouse tests has been traced to its poor ability to survive under field conditions (24,56). For example, a strain of Erwinia herbicola was indicated as a promising biological control agent because, besides being able to antagonize ice nucleation-active bacteria, it could colonize corn plants in the field, therefore decreasing frost injury on those plants (89).

The mechanisms of antagonism reported for saprophytic bacteria against bacterial pathogens have been related to acid production (30,39,74), competition for sites or nutrients (22,59,62,74), antibiotic production (17,30,77,85,86), parasites (78), or induction of host resistance (22,40,66,88). The purpose of this work was to determine the ability of bacterial cultures, selected in greenhouse experiments, to control BLBSR of corn under field conditions and to investigate their survival ability in the field. An attempt was also made to explain the mechanism of antagonism involved in disease control.

Materials and Methods

Evaluation of Antagonists in the Field

The experiment was carried out at the Horticultural Unit, University of Florida, Gainesville. Ten 100-m rows of sweet corn cv. Merit were planted on September 22, 1985. The spacing between rows was 0.80 m and the seeds were planted with a planter regulated to deliver one or two seeds per 20 cm of row. Soil preparation, fertilization, and herbicide applications were performed according to recommendations for the crop in Florida (81). The plants were sprayed every three or four days after emergence with a Carbaryl insecticide for control of leaf-damaging insects, especially the corn earworm [*Heliothis zeae* (Boddie)].

The factorial experiment used consisted of a split plot design in three blocks. The two best antagonists found in the greenhouse experiments, *Pseudomonas fluorescens* F-11 and *Bacillus* sp. U-46, and the water and streptomycin controls, were the main plots consisting of two 30-m rows. The subplots were 10 m long and consisted of the time (0, 12, and 48 hours) between antagonist (or control) application and inoculation with *P. avenae*.

Approximately 2 ml of a suspension containing 5x10^7 cfu/ml of the antagonists, streptomycin at 100 ppm, or sterile tap water were applied per plant. The antagonists and the controls were sprayed in the afternoon with a hand sprayer for approximately one second per plant with the jet directed to the whorl. Two identical sprayers were used, which were washed between two applications with 0.5% sodium hypochlorite, thoroughly rinsed with deep-well water, and rinsed with sterile tap water. The plants were at the seven-leaf stage at the beginning of the experiment.

The inoculum was prepared by growing *P. avenae* in plates of nutrient agar for 48 hours at 30 C, suspending the colonies in sterile tap water, and adjusting the concentration with a spectrophotometer at 600 nm. Plants were inoculated with *P. avenae* by spraying with approximately 1 ml per plant of a suspension with 10^7 cfu/ml of *P. avenae* and 0.01% Tween 20. A hand sprayer was used and the jet was directed toward the plant whorl. Disease intensity was assessed on the leaves that were one-half to two-thirds unfurled at the time of inoculation. Thirty to 40 leaves were harvested from the center section of both rows of each treatment in each block. Disease severity was assessed according to a 0-5 scale, with 0 representing no visible symptoms and 5 representing death of the plant. The intermediate values were based on the estimation of the percentage of blighted leaf area.

Survival of Antagonists in the Field

Rifampin-resistant mutants of strains F-11, F-24, and U-46 were used in this experiment. Strains F-11 and F-24 were isolated from different corn plants in Gainesville, Florida; both belong to the same biovar of *P. fluorescens*. Culture U-46 is a *Bacillus* species. Cultures F-11, F-24, and U-46 were the best antagonists against *P. avenae* in greenhouse tests. To obtain the rifampin-resistant mutants, the parent cultures were grown in 5 ml of TSB for 24 hours at 30 C in test tubes constantly shaken at 100 rpm. Spontaneous mutants were isolated by plating the cultures in TSA containing 100 µg/ml of rifampin (Sigma Chemical Company, St. Louis, MO). Stability of each mutant was tested by growing single colonies in four subcultures in antibiotic-free Trypticase soy broth and then counting the number of cells on TSA with and without 100 µg/ml of rifampin. Mutants were selected that were identical in colony numbers in plates with and without the antibiotics.

The antagonists were sprayed in the late afternoon, each on five plants of sweet corn cv. Merit. The plants were at the six-leaf stage and were randomly selected in a 20x100 m field. Approximately 1.5 ml of a suspension of 10^8 cfu/ml was applied to the whorls of plants with a Chromist spray unit (Gelman Instrument Co., Ann Arbor, MI).

Samples of approximately 0.5 ml of fluid were withdrawn from the whorls of the same plants with a graduated disposable transfer pipet (Fisher Scientific, Pittsburgh, PA) at 0, 12, 36, 84, and 204 hours after application of the antagonists. The pipets were sealed and transported to the laboratory in a cooler and the samples were processed in the same day. The populations of applied and total bacteria were determined through the dilution-plate method in KMB medium containing 100 μg/ml rifampin and on TSA, respectively. Average daily temperature and rainfall data were obtained from a weather station located nearby the experimental plot.

Mechanism of Antagonism

The production of diffusible inhibitory substances was evaluated for cultures F-11, F-18, F-22, U-132, and ABG 4000 on half-strength TSA and on KMB media. Cultures F-11, F-18, and F-22 were isolated from the whorl of corn plants at the Horticultural Unit, University of Florida, Gainesville, and previously identified as *Pseudomonas* fluorescens based on tests proposed by Palleroni (72). Culture U-132, identified as P. putida (72), was isolated from the leaf surface of a corn plant in an experimental plot in Gainesville. Culture ABG 4000, a Bacillus species previously known to be antagonistic to P. avenae in vitro, was kindly provided by Abbott Laboratories, North Chicago, IL. The TSA plates were spotted in the center with a suspension of 10^8 to 10^9 cfu/ml of each antagonist by using a transfer loop with about 1 mm in diameter. The plates were incubated for 48 hours at 30 C and then sprayed with a chromist sprayer for one second with a suspension of 10^6 cfu/ml of P. avenae. The plates were then reincubated at 30 C for 24 hours. The KMB plates were spotted the same way, incubated for 48 hours at 30 C, sprayed with ferric chloride (2 mg/ml, 0.2 ml/plate), trypsin (2.5 mg/ml, 0.2 ml/plate), or a mixture of both. Ferric chloride and trypsin were applied to determine the production of siderophores (71) or bacteriocins (90), respectively. These plates were kept for two hours at room temperature and then sprayed with a suspension of P. avenae as before. Plates of KMB without ferric chloride and trypsin were used as control. Antibiotic production was estimated by measuring the diameter of the zone of inhibition, which was given by the total diameter of the zone minus the diameter of the bacterial colony.

Antagonism of F-11 through acid production was tested by measuring the pH of the medium before and after growing the antagonist for 24 hours in tubes containing 5 ml of either nutrient broth or filtered fluid from corn whorls.

The production of inhibitory compounds was also evaluated by measuring the growth of P. avenae in the filtered fluid obtained from the whorl of corn plants (fluid filtrate), in the filtered fluid filtrate where F-11 had grown for 24 hours (F-11 filtrate) and in the fluid filtrate in the presence of F-11. Filtrations were made with disposable 0.20 µm sterilization filter units (Nalge Company, Rochester, NY). Approximately 0.1 ml of a suspension containing 10^5 cfu/ml of a rifampin-resistant mutant of strain 83-1 of P. avenae was pipeted into test tubes with 1 ml of fluid filtrate, F-11 filtrate, or fluid filtrate plus 50 µl of a water suspension of F-11 containing approximately 10^8 cfu/ml. The treatments were replicated three times. The growth of P. avenae was measured through the dilution plate technique in TSA containing 100 µg/ml of rifampin at 0, 2, 6, 16, and 24 hours of incubation at 30 C under constant agitation at 100 rpm.

Results

Disease Control in the Field

A significant interaction was found between time of application and type of treatment in the field plots, i.e., the effect of a treatment was affected by the time between application of that treatment and the inoculation with *P. avenae*. The best control of BLBSR was provided by the antagonist F-11; this treatment was significantly different from the other treatments at all times, except from streptomycin applied at time 0 (Fig. 5-1). Streptomycin controlled the disease only when applied at the time of inoculation and antagonist U-46 was not effective regardless the time of application.

Survival of Antagonists in the Field

Populations of antagonist U-46 declined rapidly and could not be detected at 84 hours after it had been applied in the corn plant whorls. Conversely, antagonists F-11 and F-24 persisted in significant numbers on the plants for up to eight days following application (Fig. 5-2). The utilization of rifampin-resistant mutants allowed the detection of a small number of cells of the test species in relation to the total population on the plant (Fig. 5-3). The selected rifampin-resistant mutants of F-11, F-24, and U-46 were as effective as the wild types in controlling BLBSR in corn plants in the greenhouse and, apparently, no competitive disadvantage was associated with the mutations.

Mechanism of Antagonism

Cultures F-11 and F-18 produced iron-regulated antibiotics, i.e., antagonism in vitro was due to iron competition by the production of siderophores. Whenever

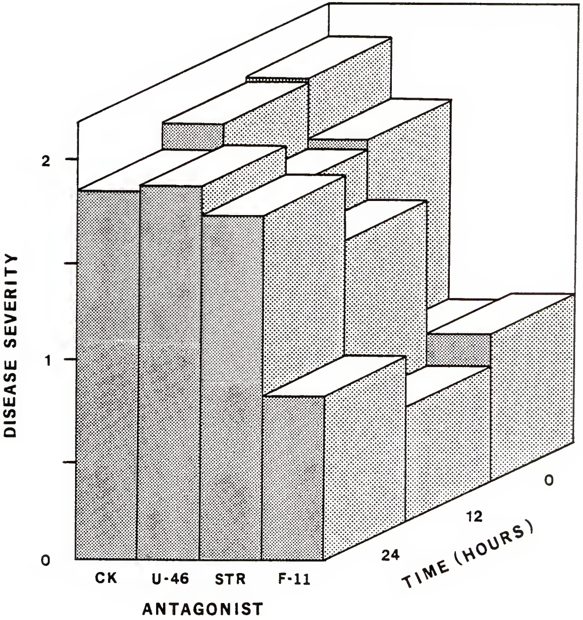


Fig. 5-1. Control of bacterial leaf blight and stalk rot on sweet corn cv. Merit in the field by application of antagonists Bacillus sp. U-46 and Pseudomonas fluorescens F-11 at 0, 12, and 24 hours before inoculation with P. avenae. Streptomycin (100 ppm) and water sprays were used as controls. Each value represented is an average of the disease severity on three replications, each with 30 to 40 leaves.

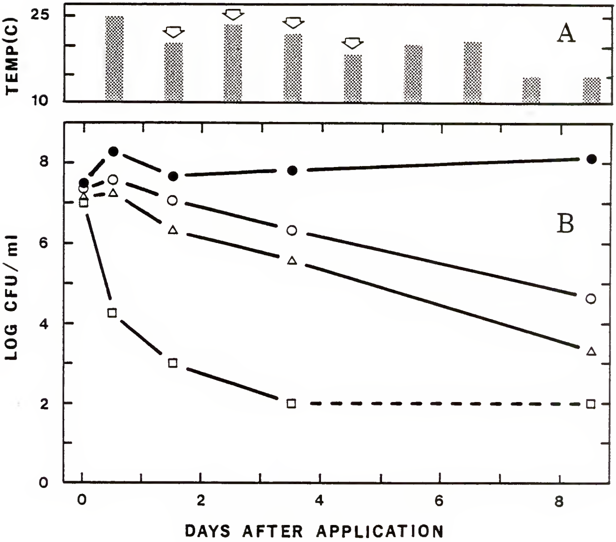


Fig. 5-2. Survival in the field of rifampin-resistant mutants of antagonists Pseudomonas fluorescens F-11 (o), P. fluorescens F-24 (△), and Bacillus sp. U-46 (□) in the whorl of sweet corn cv. Merit (B). Numbers represented are average of readings in five plants. Total bacterial (●) is an average of all plants sampled in a given day. Dashed line indicates the limit of detection for the three antagonists. The daily average temperature for the period and the day when rainfall occurred (arrows) are indicated (A).

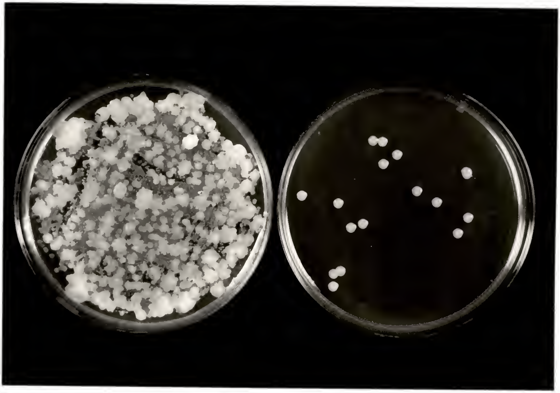


Fig. 5-3. Bacterial growth on King's medium B without (left) and with (right) 100 µg/ml of rifampin. Identical volumes of the same suspension were spread on both plates. Only the rifampin-resistant mutants grew on plates with the antibiotics.

iron was added to the medium, the antibiotic effect was eliminated (Table 5-1). The addition of trypsin did not reduce antibiosis in any case, indicating that none of the cultures produced bacteriocins against P. avenae. Addition of iron did not affect the diameter of the zones of inhibition produced by cultures F-22, U-132, and ABG 4000, indicating that, for those antagonists, antibiosis was not related to siderophore production. Culture ABG 4000 produced a zone on TSA but not in KMB, probably because of its poor growth in KMB.

Antagonism of F-11 apparently is not due to acid production, since the pH of the fluid filtrate and the nutrient broth remained approximately the same, respectively 6.3 and 6.8, after F-11 had grown in them for 24 hours.

Populations of P. avenae increased in both fluid filtrate and in F-11 filtrate, but decreased in the fluid filtrate in the presence of F-11 (Fig. 5-4). Approximately 50% of the colonies of P. avenae which grew by plating the suspension in which this pathogen had grown in the presence of F-11 for 24 hours were of a rougher and larger type as compared to the fluidal types which grew in the other substrates. If both bacteria were grown together for 16 hours or less, only colonies of the fluidal type were obtained.

Table 5-1. Diameter of zones of inhibition of *Pseudomonas avenae* by selected bacterial cultures in different media.

Medium	Antagonist				
	F-11	F-18	F-22	U-132	ABG 4000
TSA	0	0	18	22	15
KMB	11	18	19	26	0
KMB + Fe	0	0	19	25	0
KMB + T	11	16	18	23	0
KMB + Fe + T	0	0	20	23	0

Numbers represent the average of two measurements.
Fe = Ferric chloride; TSA = Trypticase soy agar; T = Trypsin; KMB = King's medium B.

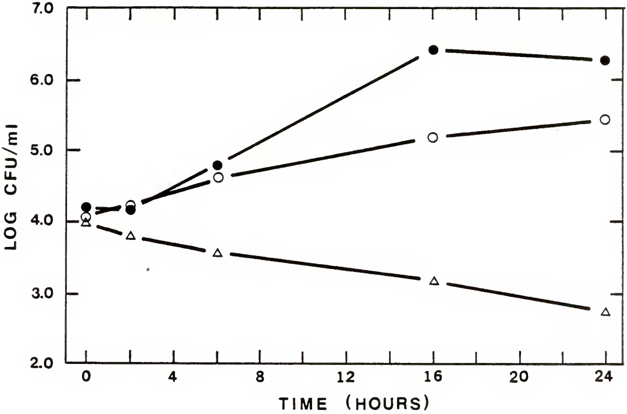


Fig. 5-4. Multiplication of a rifampin-resistant mutant of Pseudomonas avenae strain 83-1 in the filtrate of fluid from the whorl of corn plants (fluid filtrate) (●), in the refiltered fluid filtrate where P. fluorescens F-11 had grown for 24 hours (F-11 filtrate) (o), and in the fluid filtrate in the presence of F-11 (△).

Discussion

Antagonist F-11 significantly reduced bacterial leaf blight and stalk rot when it was applied to sweet corn seedlings at 0, 12, and 24 hours before inoculating them with *Pseudomonas avenae*. Disease control was comparable to that obtained by applying streptomycin at 100 ppm immediately before inoculation. Application of streptomycin at 12 and 24 hours before inoculation did not control the disease. This is in agreement with the findings of Gitaitis (36) in that antibiotics protects corn plants against *P. avenae* only for a short period. This is due to the rapid growth of the corn plant, which causes a constant exposure of new and unprotected tissues to the pathogen. Conversely, there was a redistribution of the protecting factor produced by the antagonist *P. fluorescens* F-11 which may have been provided by its fast multiplication and long survival in the corn whorl. This was indicated by the recovery in large numbers of the rifampin-resistant mutant of the antagonist even at eight days after its application to plants in the field. The data represented in Fig. 5-2 would be even more striking if it were possible to account for the dilution of the populations of F-11 which might have occurred due to rain, mist, dew, and guttation water accumulated in the whorl. Furthermore, only the first two samplings were withdrawn from the same site in the whorl where the antagonist had been applied. Subsequent samples were taken from the space between newly formed leaf blades. Therefore, the population of F-11 recovered was probably not constituted of cells originally applied, but of their progenies. It needs to be determined whether populations of the antagonist found in these new tissues at different times after application are large enough to inhibit the infection by natural levels of incoming propagules of *P. avenae*. This would indicate how frequently the antagonist needs to be applied in order to provide significant disease control. Observations in a field adjacent to the experimental plots indicated that antagonist F-24, which was undistinguishable from F-11 in all tests performed to identify the two cultures, was as efficient as F-11 in controlling BLBSR.

Antagonist U-46 was not as effective as F-11 and F-24 in controlling BLBSR in the field. This may be so because it did not survive well in the whorl of corn plants. The decrease in the population of U-46 was close to 10,000 times in only 36 hours.

The mechanism of antagonism involved in disease control by F-11 apparently was not due to acid production, since there was no alteration in the pH of the medium after F-11 had grown in it for 24 hours.

Even though F-11 and *P. avenae* are not closely related, the possible production of bacteriocin by F-11 was tested. The failure of a protease (trypsin) to suppress the inhibition zone in KMB indicates that antibiosis was not due to bacteriocins which, in their majority, contain protein as an essential constituent (90). Antagonist F-11 also did not produce any zone of inhibition to P. avenae in iron-rich media such as TSA. These findings are in agreement with Lindow (61), who found that only 58% of 88 bacteria antagonistic to P. syringae on leaf surfaces produced compounds inhibitory to P. syringae on culture media.

Zones of inhibition produced by F-11 on KMB plates sprayed with P. avenae were due to siderophore production by F-11, as indicated by elimination of the antagonistic effect with the addition of iron to the medium. The production of siderophores by fluorescent pseudomonads has been postulated as a mechanism of antagonism for soilborne pathogens (51,52,83). The role of siderophores in disease suppression of foliar bacterial diseases, however, is not clear, although it has been suggested in at least one case (17).

Antagonism of F-11 to P. avenae in planta is not believed to be due to siderophore production, since the pathogen grew well in the fluid filtrate where F-11 had grown for 24 hours. Deprivation of the medium from iron to inhibitory levels would have prevented the growth of the pathogen if iron were an essential nutrient for its growth and if siderophores had been produced in sufficient amounts to chelate most of it. Furthermore, other epiphytic fluorescent pseudomonads, which apparently produced larger amounts of siderophores than F-11 in KMB, completely failed to control the disease in the greenhouse. A definitive test for establishing the need of siderophores for antagonism would be the application of iron to plants just prior to inoculation, but after the antagonist has been applied. Failure to reduce the antagonistic effect by the addition of iron would indicate that antagonism is not due to siderophore production.

The increase in populations of *P. avenae* in corn-fluid filtrate is in agreement with data presented by Gitaitis (36), who found that the corn fluid contains essential compounds for the multiplication of *P. avenae*. The fluid filtrate also allowed abundant growth of *P. fluorescens*, as could be observed by the cloudiness of the suspension which developed after 24 hours of incubation at 30 C. *Pseudomonas avenae* also multiplied in the F-11 filtrate, indicating that no stable antibiotics were produced by the antagonist in significant amounts during the period of 24 hours of incubation. The lower numbers of *P. avenae* in the F-11 filtrate, as compared to the fluid filtrate, is probably due the partial exhaustion of nutrients in the medium.

The slow decrease in the populations of *P. avenae* when in the presence of F-11 is apparently not large enough to solely explain the degree of disease suppression observed in all the experiments.

The mutants of the rifampin-resistant mutant of *P. avenae* which appeared when the pathogen was grown in the fluid filtrate in the presence of F-11 was analyzed for the presence of plasmids (unpublished data). This test was performed in order to determine if genetic exchange was occurring, which could interfere with the virulence of the pathogen. No plasmids were detected in any of the wild type, the rifampin-resistant mutant, or the rough mutant of rifampin-resistant mutant of *P. avenae* which formed larger colonies. No plasmids were detected in F-11 either. Furthermore, the two mutants and the wild type of the pathogen induced the same amount of disease in sweet corn plants inoculated in the greenhouse. It was concluded that antagonism by F-11 is not because of an impairment of the virulence of the pathogen.

The above evidences suggest that F-11 secretes volatile or unstable substances which are inhibitory to *P. avenae* or which stimulate the resistance of the plant. It is important to consider here that the efficacy of an antagonist might not be attributed to one property only, such as production of inhibitory substances. A number of biological processes, as with pathogenesis, may determine the ability to control disease (79).

Strains F-11 and F-24 of P. fluorescens were shown to be promising biocontrol agents. However, further tests are necessary before their application can be recommended for the control of BLBSR on corn at the farm level. Some of the questions that must be resolved are (a) by what mechanism(s) the antagonist is limiting disease development; (b) how the microenvironment in the plant, especially nutritional, could be changed in order to favor the establishment of the antagonist; (c) what is the effect of Tween 20 on disease development in the field and what effect it has on the efficiency of the antagonist; (d) how disease control in seedlings correlates to control of the shank-rot stage of the disease; (e) how efficient is the biocontrol agent in controlling other corn diseases, especially the stalk rots caused by Erwinia spp., which also start in the whorl; (f) what control is achieved by the application of the antagonist under natural inoculum densities in the field; (g) how feasible is the commercial production of a formulation and what are the best ways to apply the product in the field, taking into consideration the age of the plant; and (h) what consequences the application of large numbers of bacteria in the field would have to the microbial population and to the environment.

CHAPTER SIX
SUMMARY

Bacterial leaf blight and stalk rot (BLBSR) of corn, caused by Pseudomonas avenae, can be a serious disease on sweet corn if environmental conditions are favorable for disease development. Although there are sweet corn genotypes which are resistant to the disease, some of the most popular cultivars are very susceptible to BLBSR and sustain considerable losses. Besides resistance, no other control measure is available for controlling BLBSR. Even if suitable bactericides were available, they probably would not be effective against the disease. Rapidly growing tissues, which are most susceptible to BLBSR, cannot be continuously protected with the bactericide, unless the bactericide has the property of redistribution in the plant.

Bacteria are found as epiphytes on the surface of most, if not all, plant species. The interactions among saprophytic bacterial epiphytes and plant pathogenic species in the soil or in the plant phylloplane often result in a suppression of plant disease. The proposed mechanisms by which bacteria antagonize bacteria are competition for sites or nutrients, parasitism, antibiosis, or stimulation of host resistance.

This study was intended to determine the feasibility of using bacterial epiphytes isolated from corn plants to control BLBSR on sweet corn. The conclusions from this study are summarized as follows:

1. The pathosystem and the methodology used were adequate for biological control studies for the following reasons: A) There is a close contact between the putative antagonist and cells of _P. avenae_ in the whorl of the corn plant before infection takes place. B) Disease control by an antagonist can be assessed in seedlings. C) The inoculation technique is quick, easy, and reliable; and the latent period of the disease is short. Therefore, large number of bacterial cultures can be screened for antagonism in a relatively short period of time. And D) Sampling for populations in plants in the field does not need to be destructive, thus allowing quantitative and qualitative studies on the succession of bacterial populations within the same plant.

2. Epiphytic bacterial populations measured in the fluid collected in the whorl of corn plants in the field were found to be not normally, but lognormally distributed. This means that samples for quantitative and qualitative studies of bacterial populations in the whorl

of corn plants should not be bulked, but analyzed separately for each plant.

3. From 179 cultures of epiphytic bacteria collected from corn plants in three locations in Florida, 34 were antagonistic in vitro to *P. avenae*. Among these, 29 were fluorescent pseudomonads, indicating that special attention should be given to this group of bacteria in future searchs for antagonists.

4. The number of antagonists collected from a sweet corn cultivar which is resistant to BLBSR, Silver Queen, did not differ from the number collected from the susceptible cultivar Merit, suggesting that the different genotypes did not influence the bacterial numbers.

5. There was no correlation between antagonism in vitro, which was measured by the production of zones of inhibition to *P. avenae* in petri dishes, and ability to control BLBSR in corn plants in the greenhouse.

6. The addition of 0.01 to 0.10% of Tween 20 to the suspension of *P. avenae* prior to inoculation of plants in the greenhouse greatly reduced the experimental error by reducing the number of plants that escaped disease.

7. *Pseudomonas fluorescens* F-11, *P. fluorescens* F-24, and *Bacillus* sp. U-46 were found consistently to suppress development of BLBSR on sweet corn plants in the greenhouse.

8. Infectivity titration of _P. avenae_ on greenhouse-grown plants of sweet corn, either protected or not protected with _P. fluorescens_ F-11, indicated that the control achieved by the application of the antagonist was equivalent to reducing the inoculum dose 1000 or 6025 times, respectively, when disease was assessed in terms of incidence and severity, respectively. A better correlation between inoculum dose and disease intensity was achieved when disease severity was assessed.

9. In field tests, _P. fluorescens_ F-11 controlled BLBSR on sweet corn as effectively as streptomycin at 100 ppm when the treatments were applied to the plants at the time of inoculation with _P. avenae_. When they were applied 12 or 24 hours before inoculation, however, streptomycin did not control the disease, whereas the antagonist reduced BLBSR severity in more than 50% in relation to the water-sprayed control. _Bacillus_ sp. U-46 did not affect disease development in plants in the field at any time of application.

10. _Pseudomonas fluorescens_ F-11 survived well in corn plants in the field and could be consistently recovered from the top funnel of plants even eight days after its application in the whorls. Conversely, _Bacillus_ sp. U-46 could not be recovered 84 hours after it had been applied to the plants. Disease control by _P. fluorescens_ F-11 is apparently related to its ability to colonize the

whorl and redistribute to newly formed tissues, thus protecting them against BLBSR.

The mechanism by which _P. fluorescens_ F-11 antagonizes _P. avenae_ has not been fully determined. Evidences were found that it is not related to production of acid or stable antibiotic substances (including siderophores), to impairment of the virulence of the pathogen, to competition for nutrients with the pathogen at the infection court, or to direct parasitism. Therefore, antagonism is presumed to be through the production of unstable or volatile antibiotic substance(s), through stimulation of host resistance, through competition for infection sites in the whorl, or a combination of any of these factors.

The results obtained in this study indicate that aerial parts of a corn plant may harbor bacteria that can be utilized to control BLBSR of corn and probably other bacterial diseases of corn. The two strains of _P. fluorescens_ that most efficiently controlled the disease under field conditions are believed to be valuable material which, after further evaluation, can be utilized in an integrated pest management program for sweet corn in regions where BLBSR is a problem.

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BIOGRAPHICAL SKETCH

Carlos Alberto Lopes was born in Vicosa, MG, Brazil, on November 10, 1950. He graduated from high school in Colegio de Vicosa in 1968. He received the degree of Bachelor of Science in Agriculture in 1973 from Universidade Federal de Vicosa. In 1974, he was employed by the Brazilian Agricultural Research Corporation (EMBRAPA). He received his Master of Science in plant pathology at the University of Wisconsin-Madison in 1977. From 1977 to 1983, he worked in Brasilia at the CNP-Hortalicas/EMBRAPA with fungal and bacterial diseases of vegetable crops. He came to the University of Florida in the spring of 1983 to pursue the degree of Doctor of Philosophy in the Plant Pathology Department. Upon his return to Brazil, Carlos will resume his work in EMBRAPA.

Carlos is married to Silvia and has a daughter, Mariana.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert E. Stall

Robert E. Stall, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Raghavan Charudattan
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James O. Strandberg

James O. Strandberg
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Mark J. Bassett

Mark J. Bassett
Professor of Horticultural Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1986

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School